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## Molecular Targets in Head and Neck Cancer

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Molecular Targets in  
Head and Neck  
Squamous Cell Carcinoma

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**Abstract:****Background:**

Head and neck cancer (HNSCC) affects 650,000 people annually. Laryngeal cancer (LSCC) and oropharyngeal cancer (OPSCC) are amongst the commonest sub-types.

For other cancers e.g. breast cancer, personalised treatments based on tumour markers have improved patient survival. With the exception of human papilloma virus (HPV); there are no clinically utilised biomarkers in HNSCC.

Insulin growth factor receptor 1 (IGF-1R) and HPV are promising molecular markers in LSCC and OPSCC respectively. This thesis investigates the use of IGF-1R as a marker of radiotherapy resistance in LSCC and evaluates HPV detection in patients with OPSCC.

**Aims:**

- To assess IGF-1R as a marker of radiotherapy resistance in LSCC.
- To determine the diagnostic accuracy of salivary PCR to detect HPV in patients with OPSCC.

**Methods:**

Immunohistochemistry (IHC) was used to compare IGF-1R levels between patients with LSCC achieving long-term remission and experiencing recurrence after radiotherapy. LSCC cells were used to create and interrogate an in vitro model of radiation resistance.

Following the completion of a systematic review on HPV testing in OPSCC, a diagnostic accuracy study was performed to determine the sensitivity and specificity of saliva testing for HPV in OPSCC.

### **Results:**

IGF-1R levels are higher in radioresistant LSCC and increase following radiotherapy. IGF-1R inhibition appears to be more effective at limiting cell survival in cells with IGF-1R overexpression.

The sensitivity and specificity of saliva testing when compared to p16 IHC and HPV DNA in situ hybridisation is 72.2% and 90%.

### **Conclusions:**

Elevated IGF-1R appears to associate with previous radiotherapy and radiotherapy resistance in LSCC. Treatments accounting for IGF-1R status, or molecular therapies targeting this receptor, may have merit in patients whose tumours overexpress IGF-1R.

Saliva testing for HPV is a promising alternative to p16 IHC performed on tumour tissue. In selected patients, this might avoid the need for surgical biopsies and expedite treatment.



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“And whatever of blessings and good things you have, it is from God” [al-Nahl16:53].

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## **Introduction: Molecular Targets in Head and Neck Cancer**

Head and neck squamous cell carcinoma (HNSCC) is the 6<sup>th</sup> commonest cancer worldwide affecting 650,000 people each year [1]. Two of the most commonly affected sites are the larynx and oropharynx with 2,315 [2] and 2,303 [3] new cases diagnosed each year in the United Kingdom (UK). Both laryngeal squamous cell carcinoma (LSCC) and oropharyngeal squamous cell carcinoma (OPSCC) are treated with surgery or chemoradiotherapy.

Research into the field of personalised medicine has shown that molecularly distinct subtypes of common cancers exist. These findings have had implications on prevention, diagnosis, prognosis and treatment in numerous solid organ tumours [4].

Examples include the discovery that mutations in the BRCA gene give a 45-65% chance of developing breast cancer by the age of 70. This has led many patients to opt for prophylactic mastectomy or hormone suppression to prevent breast cancer. More recently the presence of BRCA1/2 has also shown the ability to predict response to anti-PARP therapy in ovarian cancer [5]. In metastatic or non-resectable malignant melanoma the introduction of Vemurafenib in patients with an activating mutation in the BRAF oncogene has led to increases in overall and progression free survival of 3.9 months and 5.3 months respectively in comparison to standard treatments [6].

Treatments for HNSCC have not kept pace; there is a need to bring personalised medicine into the care of these patients. For LSCC and OPSCC the choice, number and regime of treatments offered is guided by disease stage, patient co-morbidity, patient choice and clinical expertise. It is only very recently that the presence of human

papilloma virus (HPV) has shown a potential role in the management of patients with OPSCC [7] [8].

### Laryngeal Cancer

Laryngeal squamous cell carcinoma (LSCC) affects four times as many men as women and is caused by tobacco smoking and alcohol consumption, these have a synergistic effect on the onset of disease [7]. The clinical presentation of LSCC depends on the sub-site of the larynx affected. Glottic disease, which affects the true vocal cords typically presents early with patients experiencing a hoarse voice. Subglottic or supraglottic disease usually presents later with dysphagia, odynophagia, neck masses or rarely airway obstruction.

In the UK there has been an approximate 20% reduction in new diagnoses and deaths from laryngeal cancer over the past 2 decades [7]. These observations may relate to the success of smoking cessation interventions [9] or earlier diagnosis through increased clinician awareness and earlier specialist referral [10].

There are no clinically utilised biomarkers for LSCC and when compared to other solid organ tumours e.g. breast, lung and prostate there have been relatively few new treatments to improve survival [11]. Instead there has been a focus on organ preservation and function through targeted radiotherapy e.g. intensity-modulated radiotherapy and minimal access surgery e.g. transoral laser microsurgery [12].

There is clearly a need for new molecular therapies and biomarkers for LSCC and in the age of personalized medicine, agents targeting the epidermal growth factor receptor

(EGFR) appear promising for both [13]. EGFR is overexpressed in more than 80% of HNSCCs and increased expression is associated with poorer prognosis, increased loco-regional recurrence and increased resistance to radiotherapy [14,15]. A better understanding of how EGFR interacts with other receptor tyrosine kinases (RTKs) e.g. IGF-1R may provide the key to solving the challenges of treatment resistance.

Clinically the only new agent to gain National Institute for Health and Care Excellence (NICE) approval for LSCC in the past decade is Cetuximab [16]. In randomised controlled trials, this monoclonal antibody to EGFR, when combined with radiotherapy vs. radiotherapy alone has shown improved local-regional control and 5 year survival of 24.4 months and 45.6% vs. 14.9 months and 36.4% respectively [17,18]. Unfortunately there is a short-term response to treatment due to acquired resistance thereby limiting its clinical application [19].

Colorectal cancers also overexpress EGFR and were initially thought to develop treatment resistance to Cetuximab. The discovery that only colorectal tumours expressing wild-type KRAS responded to this treatment led to the licensing of Cetuximab as a new treatment in this group of patients. We now know that Cetuximab improves quality of life and doubles overall and progression free survival when compared to supportive care only for patients with wild type KRAS and colorectal cancer. As a result KRAS testing is now routine for patients with colorectal cancer [20].

As with other solid organ tumours it is hoped that EGFR and associated receptor tyrosine kinases e.g. IGF-1R may prove to be useful biomarkers or molecular targets for future treatment regimes in LSCC. Better appreciation of their interaction and

expression in vitro and in vivo may help to identify new sub-groups of patients likely to respond to or fail conventional treatment regimes.

### Oropharyngeal Cancer

Oropharyngeal squamous cell carcinoma (OPSCC) affects the palatine tonsils or tongue base and is conventionally associated with alcohol consumption and smoking. More recently a new sub-type has emerged associated with human papilloma virus (HPV) in younger healthier non-smokers. Despite a reduction in smoking and alcohol consumption, the UK incidence of OPSCC doubled between 1990 and 2006 and then again between 2006 and 2010 [8]. The exponential rise was attributed to HPV associated OPSCC (HPV+ve). HPV+ve OPSCC is set to overtake the incidence of HPV associated cervical cancer by 2020 [21] and is a major health epidemic in the western world [22].

Patients with OPSCC can remain asymptomatic for prolonged periods before presenting with otalgia, dysphagia, odynophagia, neck masses and rarely breathing difficulties. The prognosis and response to treatment for conventional or HPV negative (HPV-ve) OPSCC is very different to HPV+ve disease. Local-regional treatment failure and 5 year survival is reported at 13% and 82% for HPV+ve OPSCC whilst it is 42% and 35% in HPV-ve disease [23].

HPV is the only biomarker of clinical relevance for patients with HNSCC but is only clinically useful for patients with OPSCC. The presence of HPV can predict prognosis, guide treatment decisions and determine suitability for entry into de-escalation clinical trials [24].

HPV testing is considered the standard of care for patients with OPSCC and is performed using polymerase chain reaction (PCR), DNA in situ hybridisation (ISH) and p16 immunohistochemistry (IHC) [25]. All of these tests are limited by the need for invasive surgical biopsies that take time to collect and are associated with morbidity.

The ideal test for HPV in patients with OPSCC would be non-invasive, economical and taken at the point of first patient contact. It could form part of screening/monitoring programmes for individuals with OPSCC and be performed by general practitioners at the time of specialist referral. This might shorten time to diagnosis and treatment; improve patient satisfaction and overall mortality. Recent tests have emerged with the potential to use saliva to detect oropharyngeal HPV, however, they are yet to be validated in the context of OPSCC [26]. If proven accurate saliva testing could replace the need for surgical biopsies for HPV in OPSCC.

## **Chapter 1: Insulin Like Growth Factor Receptor 1 (IGF-1R) and Radiotherapy**

### **Resistance in Laryngeal Cancer**

#### **Abstract:**

#### **Background:**

Salvage surgery is the only option for radiotherapy failure in laryngeal squamous cell carcinoma (LSCC), but is associated with high morbidity. There is a need to identify biomarkers of radio resistance, to inform treatment decisions. Epidermal growth factor receptor (EGFR) associates with radiotherapy resistance in head and neck cancer (HNSCC), and insulin-like growth factor receptor type 1 (IGF-1R) correlates with radiotherapy resistance in other tumour types. We recently reported that IGF-1R associates with advanced T-stage, HPV negativity and adverse survival in HNSCC. Here, we evaluated IGF-1R and EGFR in predicting radiotherapy failure in LSCC.

#### **Aims:**

To assess IGF-1R and EGFR expression in patients treated with radiotherapy for LSCC and compare receptor levels in patients experiencing radiotherapy without known recurrence vs. those experiencing radiotherapy failure.

#### **Methods:**

We scored membrane, cytoplasmic and total (membrane plus cytoplasmic) EGFR and IGF-1R using immunohistochemistry on biopsies and salvage laryngectomies from 63 LSCC patients, including 41 treated with radiotherapy (23 radiotherapy without known recurrence, 18 local recurrences) and 22 with primary laryngectomy.

**Results:**

IGF-1R scores were higher in the biopsies of the radiotherapy failure group, with scores in the membrane of 3.07 vs. 1.0 ( $p=0.004$ ), cytoplasm 3.36 vs. 2.17 ( $p=0.18$ ) and total IGF-1R 6.43 vs. 3.17 ( $p=0.01$ ) compared with those receiving radiotherapy without known recurrence. IGF-1R expression was positively associated with increasing tumour size and increased following radiotherapy whilst EGFR expression remained static following radiotherapy. EGFR scores did not correlate with radiotherapy outcomes. Patients undergoing primary laryngectomy had higher T and N stage ( $p<0.05$ ) and higher tumour IGF-1R (8.3 vs. 3.17,  $p=0.02$ ) than those experiencing radiotherapy without known recurrence

**Conclusions:**

These results suggest that IGF-1R associates with radiotherapy resistance, higher T-stage and is up regulated following radiotherapy in LSCC. Treatments accounting for IGF-1R status, or molecular therapies targeting this receptor, may have merit in patients whose tumours overexpress IGF-1R.

## **1.1 Background:**

Over the past 20 years, unlike other solid organ tumours, 5-year survival from laryngeal squamous cell carcinoma (LSCC) in the US has remained relatively unchanged from 66% to 63% [27]. Whilst large US epidemiological studies have shown a declining incidence of 2.3% per year over the past decade[28], there will still be 13,400 new cases of LSCC diagnosed in 2016 with 3600 deaths as a direct consequence of the disease [29]. In the UK, where LSCC accounts for 1% of all cancers in men and 0.3% of cancers in women, there has been a 20% reduction in incidence and mortality over the past 2 decades [30]. These changes may be attributable to the success of smoking cessation interventions [9] or earlier diagnosis through increased clinician awareness and earlier specialist referral [10]. In the UK the overall 5 year survival is estimated at 65% [31] with approximately 2400 new cases and 800 deaths attributable to LSCC annually [30]. This makes LSCC one of the largest subgroups of head and neck squamous cell carcinoma (HNSCC).

Treatment for LSCC utilises a multidisciplinary team (MDT) approach, taking into consideration the TNM classification, patient performance status, patient preference and treatment objectives, whether palliative or curative. When curative treatment is undertaken for early stage disease (T1/2), options include radiotherapy, trans-oral laser microsurgery (TLM) or partial open laryngeal surgery. Given the absence of comparative randomised controlled trials there is insufficient evidence to determine the most effective option [32]. In the UK patients with early LSCC are usually treated with either radiotherapy with salvage laryngectomy reserved for cases of recurrence, or TLM with further surgery or radiotherapy if required [30]. Five-year survival and local control rates with radiotherapy and TLM are similar at approximately 80% [33,34].



Treatment regimes for advanced LSCC are controversial, with some authors advocating primary partial laryngeal surgery or total laryngectomy followed by radiotherapy [35,27]. Concurrent chemo-radiotherapy is widely used as the initial treatment modality aimed at preserving laryngeal function. This choice is largely influenced by the findings of the landmark Veterans Affairs (VA) [36] and subsequent Radiation Therapy Oncology Group (RTOG) [37] studies published in 1991 and 2003 respectively. The VA study demonstrated equivalent survival of 68% for patients with advanced LSCC treated with both induction chemotherapy and radiotherapy or laryngectomy followed by adjuvant radiotherapy. There was a significantly lower rate of salvage laryngectomy in T3 vs. T4 disease (29% vs. 56%  $p=0.001$ ) [36]. The RTOG study found concurrent chemo-radiotherapy was superior to both induction chemotherapy with radiotherapy and radiotherapy alone in preserving laryngeal function (88% vs. 75% vs. 70%) [37]. As a consequence of these findings patients with T3/T4 LSCC traditionally treated with laryngectomy are now frequently offered concurrent chemo-radiotherapy. Exceptions include cases where there is tumour invasion through cartilage into the soft tissues of the neck or when chemotherapy is contraindicated, for example in those over 70 years old, or with hepatic or renal impairment [30].

The overall results of laryngeal preservation for advanced stage LSCC have meant that the rates for primary total laryngectomy have declined faster than the incidence of LSCC, consistent with a trend towards nonsurgical treatment in the form of concurrent chemo-radiotherapy [38].

Although non-surgical treatments for laryngeal cancer are considered effective, treatment failure is inevitable in a proportion of individuals [39]. In early LSCC,

radiotherapy failure rates have been reported to be as high as 9%-21% in T1 and 28%-37% in T2 glottic [40,41] and 24%-30% in T1 and 25%-45% in T2 supraglottic LSCC [42,41]. In T3 and T4 LSCC non-surgical treatment failures range from 68-78.4% [43,37]. For these individuals the only remaining curative treatment is salvage partial or total laryngectomy.

Overall complication rates from salvage laryngectomy are high (42%), the commonest being pharyngocutaneous fistula (28.9%), swallowing difficulties (18.6%), stomal stenosis (17.5%), wound infection (14.1%) and bleeding (5.9%) [44]. Strategies to limit these complications include the use of local pectoralis major flaps or free tissue flaps e.g. anterolateral thigh free flap/ radial forearm free flap. Both free and local tissue transfer have an associated risk, cost and morbidity for patients [45]. In addition, overall survival is lower for patients treated with salvage laryngectomy after chemo-radiotherapy compared to radiotherapy alone, the cause for this is unclear but may relate to the long-term toxicity of chemotherapy [46].

Alongside patient factors including performance status, the TNM classification is widely adopted as the cornerstone for guiding treatment decisions in LSCC. This classification system does not predict the likely response of a tumour to radiotherapy. With a rising proportion of LSCC patients receiving chemo-radiotherapy and some authors associating this with potentially reduced overall survival [47] there is an urgent clinical need to determine predictors of radiotherapy failure in LSCC. These would allow appropriate patients to undergo primary surgery (TLM or partial/total laryngectomy), avoiding the morbidity associated with chemo-radiotherapy and salvage laryngectomy and potentially improving overall survival.

The epidermal growth factor receptor (EGFR) is overexpressed in more than 80% of HNSCCs [48,49] and is associated with poorer prognosis, increased loco-regional recurrence and resistance to radiotherapy [14,15]. When given in combination with radiotherapy, the anti EGFR monoclonal antibody Cetuximab can improve 5-year survival from 36.4% to 45.6% when compared to radiotherapy alone [18]. However Cetuximab has failed to overcome radiotherapy resistance in the majority of patients [18,1].

There is increasing evidence for cross talk between EGFR and insulin like growth factor receptor type 1 (IGF-1R) [50,51]. IGF1R over-expression has previously been linked with radiotherapy resistance in other solid organ tumours like breast [52] and cervical cancer [53] and levels are known to be elevated in HNSCC [54]. Our group has previously reported IGF-1R expression in 92% of HNSCCs. We found that overall and disease specific survival was reduced in patients with elevated IGF-1R expression in their cancers, and IGF-1R up-regulation was associated with HPV negativity and higher tumour T-stage [54]. Other than a single study [55] there is little clinical data available to assess the impact of IGF-1R expression in predicting radiotherapy resistance in LSCC.

## **1.2 Aims:**

To assess IGF-1R and EGFR expression in patients treated with radiotherapy for LSCC and compare receptor levels in patients receiving radiotherapy without known recurrence (RwR) vs. those experiencing radiotherapy failure.

### **1.3 Methods:**

A study was designed to compare total, membranous and cytoplasmic levels of IGF-1R and EGFR in patients with LSCC that had and had not responded to primary radiotherapy. The study also included measurements of the expression of EGFR and IGF-1R in patients undergoing primary and salvage total laryngectomy.

This study was conducted with ethical approval (ref no. 07/H0606/120) from the Health Research Authority (NRES committee South Central).

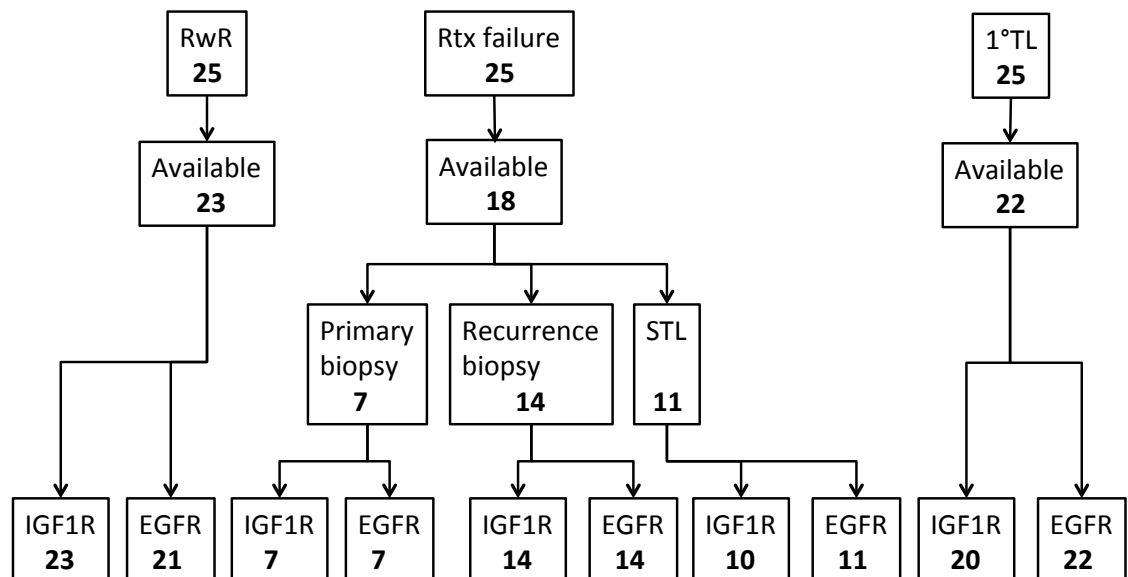
#### *1.3.1 Patient and Tumour Specimens*

All patients who had undergone salvage laryngectomy from January 2004 to January 2016 at Oxford University Hospitals NHS Foundation Trust were identified from the Oxford Head and Neck Cancer Database. There were a total of 25 such cases. Twenty-five patients treated successfully with primary radiotherapy with no known recurrence (RwR) and 25 patients treated with primary total laryngectomy during the same period were also selected at random from the database.

Diagnostic samples of formalin fixed, paraffin-embedded LSCC tissues were obtained from these cases. From the salvage laryngectomy group additional specimens were obtained where available, including biopsies of the recurrent tumour and salvage laryngectomy specimen. A consultant head and neck pathologist reviewed tissue blocks to confirm the presence of tumour tissue before 4µm whole mount sections were prepared.

Tumour samples were available from 63 of the 75 patients identified for the study. The included patient specimens were categorised into 3 groups for analysis: those experiencing radiotherapy without known recurrence (RwR) (n=23; mean follow up 4.1 yrs, range 1-5yrs), those undergoing primary total laryngectomy (n=22) and those treated with initial radiotherapy followed by local or regional recurrence and salvage laryngectomy ('radiotherapy failure'; n=18). The included samples are summarised in

**Figure 1-1.**



**Figure 1-1:** A flow diagram to represent included patient samples and available IHC results.

Abbreviations: 1°, primary; TL, Total Laryngectomy; Rx, Radiotherapy; STL, salvage total laryngectomy; SF, sample failure; EGFR, epidermal growth factor receptor; IGF-1R, insulin like growth factor receptor type 1, RwR, radiotherapy without known recurrence.

In the radiotherapy failure group there were 7 primary biopsies, 14 biopsies taken at the time of recurrence and 11 from salvage laryngectomies. Immunohistochemistry (IHC) for IGF-1R was not interpretable in 2 cases of primary laryngectomy and in 1 case of salvage laryngectomy whilst IHC for EGFR was not interpretable in 2 cases from the RwR group.

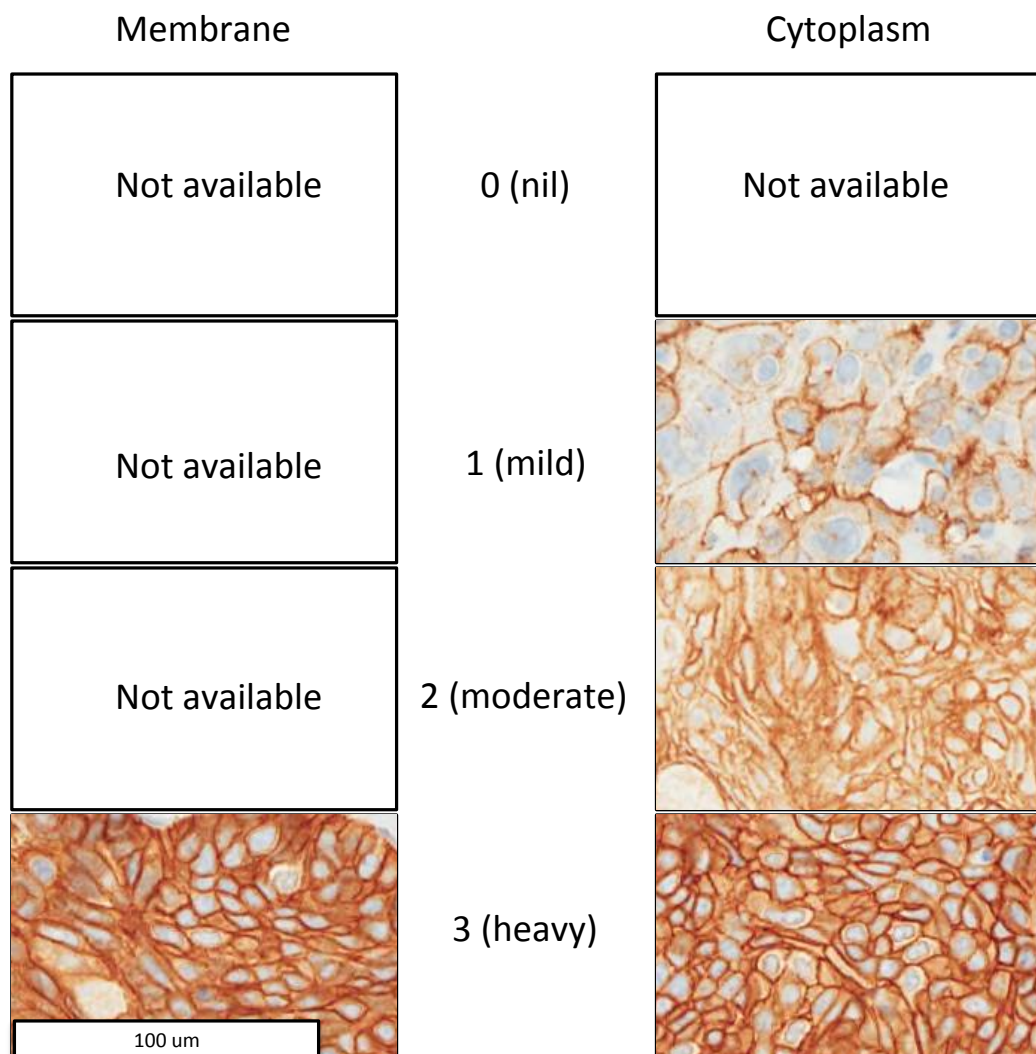
Case note review was performed for each patient to record demographic details, tumour stage (TNM staging), ASA (ASA, American Society of Anaesthesiologists Performance Status), treatment and clinical outcomes.

### 1.3.2 Immunohistochemistry

Immunohistochemistry was performed for EGFR and IGF-1R as previously described [54,56,57]. Freshly cut 4 µm whole mount sections were de waxed in Citoclear (TCS Biosciences Ltd, UK) twice for 8 minutes before being serially rehydrated in decreasing concentrations of ethanol (100%, 80%, 70% and 50%) for 2 minutes each followed by distilled water for 5 minutes. Antigen retrieval was performed in Tris/EDTA buffer (Trisma base 50nM, EDTA 2nM, pH 9) for IGF-1R and Citrate buffer (10nM citric acid, 0.05% Tween 20, pH 6) for EGFR in a decloaking chamber (DC2002, Biocare Medical) at 125°C and 85°C for 8 and 10 minutes respectively. Slides were washed in phosphate buffered saline (PBS) before blockade of endogenous peroxidase using 3% hydrogen peroxide for 10 minutes. Sections were subsequently blocked with 5% bovine serum albumin (BSA)/5% goat serum (GS) in PBS for 60 minutes. Primary EGFR antibody (no.4267, Cell Signalling Technology) and primary IGF-1R antibody (no.9750, Cell Signalling Technology) were applied respectively at 1:200 and 1:50 dilution in 5% BSA/5%GS in PBS overnight at 4°C. After 3 serial washes in PBS (5 minutes each), secondary antibody (Rabbit HRP-polymer, Menarini Diagnostics) was applied for 30 minutes followed by diaminobenzidine substrate (Envision) for 5 minutes at room temperature. Sections were counter-stained using Mayer's haematoxylin (Vector Laboratories) for 20 seconds and serially dehydrated in increasing concentrations of alcohol (50%, 70%, 80% and 100%) and Citoclear for 5 minutes each. The sections were mounted using DePex reagent (VWR International,

UK). Each staining run included control slides of sections taken from cell pellets of SKUT-1 (known to be deficient in IGF-1R and abundant in EGFR) and MCF7 (known to have relatively weaker EGFR expression and abundant IGF-1R expression)[57,58].

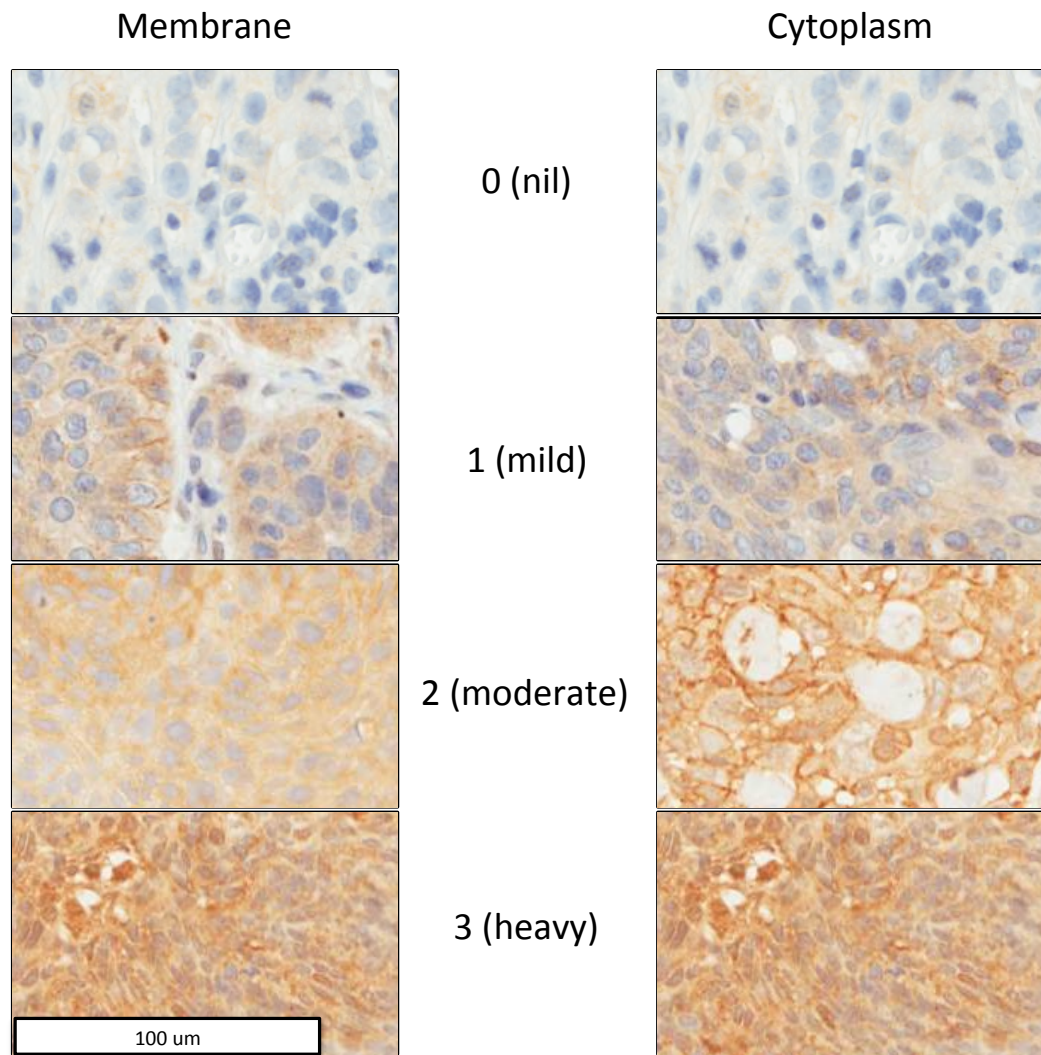
Whole mount sections were scored for membrane and cytoplasmic EGFR and IGF-1R within tumour cells only by two observers (AQ and KS). The scorers were blinded to the treatments patients had received. Any differences in score were resolved by consensus decision using a microscope with an additional teaching arm. A previously described method was used [59] to provide scores for intensity (0, no staining; 1, weak; 2, moderate; 3, heavy) and percentage of tumour involved (0%, nil; 1: 1–10% of tumour stained; 2: 11–50%; 3: 51–80%; 4: 81–100%). The two scores were multiplied to give an immunoreactive score (IRS, range 0-12) for the membrane and cytoplasm and then combined to give the total IGF-1R or EGFR score (range: 0-24). **Figure 1-2a and 1-2b** are examples of cytoplasmic and membranous staining and the scores given for EGFR and IGF-1R.



**Figure 1-2a:** Intensity scores of EGFR staining in LSCC whole mount sections.

*Examples of EGFR staining of tumour membrane and cytoplasm using IHC to illustrate patterns of intensity used to determine immunoreactivity scores. All tumour samples had 'heavy' levels of membrane EGFR expression and so nil, mild and moderate examples were not available.*





**Figure 1-2b:** Intensity scores of IGF-1R staining in LSCC whole mount sections.

*Examples of IGF-1R staining of tumour membrane and cytoplasm using IHC to illustrate patterns of intensity used to determine immunoreactivity scores.*

### 1.3.3 Statistical Analysis

All statistical analyses were pre-determined and performed using Prism 7 for Mac OS X (© 1994-2015 GraphPad Software, Inc). The demographic differences in the patient population based on primary treatment and treatment response were assessed with a two-tailed Chi-squared test. Median IRS scores determined by IHC were compared using a two-tailed Mann-Whitney U test when comparing two unpaired groups and a pairwise Mann-Whitney U test when comparing 3 or more. Association was assessed using Pearson's correlation and least squares linear regression. A result was considered significant when  $p$  was  $\leq 0.05$ .

### **1.4 Results:**

The mean age of study subjects was 66 years (range: 37- 87yrs) (see **Table 1-1**). There were 56 males (88.9%) and 7 females (11.1%). Average alcohol consumption was 18.2 units/week; there were 50 (79.4%) current or ex-smokers with an average 33.6 pack year (range: 10-120) smoking history. The majority of patients treated initially with radiotherapy presented with T1/2 disease (75.6%) whilst those treated with primary total laryngectomy had T3/4 disease (100%). There were 23 patients in the radiotherapy without known recurrence (RwR), 18 in the radiotherapy failure and 22 in the primary total laryngectomy groups respectively. In the radiotherapy failure group the average time to disease recurrence was 21.3 months (range: 4-60).

Laryngeal Cancer Treatment Group, No.(%)				
	RwR (n=23)	Rtx Failure (n=18)	Primary Laryngectomy (n=22)	P- Value (Chi <sup>2</sup> )
Sex				
Male	23(100)	16(88.9)	17(77.3)	0.053
Female	0(0)	2(11.1)	5(22.7)	
Age				
≥65	15(65.2)	9(50)	12(54.5)	0.59
<65	8(34.8)	9(50)	10(45.5)	
ASA				
1/2	21(91.3)	15(83.3)	22(100)	0.15
3/4	2(8.7)	3(16.7)	0(0)	
Smoking Status				
Never	8(34.8)	3(16.7)	2(9.1)	0.26
Current	9(39.1)	8(44.4)	13(59.1)	
Ex-smoker	6(26.1)	7(38.9)	7(31.8)	
Drinking Status				
Nil	12(52.2)	13(72.2)	10(45.5)	0.53
1-21 units/week	5(21.7)	1(5.6)	4(18.2)	
22-40 units/week	4(17.4)	2(11.1)	3(13.6)	
>40 units/week	2(8.7)	2(11.1)	5(22.7)	
T Classification				
T1/2	17(73.9)	14(77.8)	0(0)	0.001
T3/4	6(26.1)	4(22.2)	22(100)	
N Classification				
N0/1	22(95.7)	17(94.4)	14(63.6)	0.005
N2/3	1(4.3)	1(5.6)	8(36.4)	
M Classification				
M0	23(100)	18(100)	22(100)	n/a
M1	0(0)	0(0)	0(0)	
Radiotherapy Tx				
55Gy 20Fr	19(82.6)	12(66.7)	n/a	0.80
60-66Gy 30-33Fr	4(17.4)	2(11.1)	n/a	
Unknown	0(0)	4(22.2)	n/a	

**Table 1-1:** Patient Demographics and Tumour Characteristics by Primary Treatment.

*Comparisons are made using Chi<sup>2</sup> analysis. Patients with RwR and Rtx failure had similar sized tumours and patient characteristics. As expected, patients treated with primary laryngectomy had advanced stage tumours.*

Abbreviations: ASA, American Society of Anaesthesiologists Performance Status; n/a, not applicable; Tx, treatment; Gy, Gray; Fr, fractions; RwR, radiotherapy without known recurrence.

There were no statistically significant differences between the 3 groups in terms of age, sex, co-morbidity, alcohol consumption, smoking status and radiotherapy treatment regimen, although there were proportionately fewer smokers in the RwR group. Patients treated with primary total laryngectomy presented with a higher T stage and N-classification than those treated successfully and unsuccessfully with radiotherapy ( $p<0.01$ ). There were no patients with distant metastases.

#### 1.4.1 Radiotherapy without known Recurrence vs. Radiotherapy Failure

Primary biopsies from patients with RwR (n=23) were compared to primary biopsies from patients with radiotherapy failure (n=7) (**Table 1-2**).

	Membrane IRS (0-12), mean.(range)	P –value (MW-U)	Cytoplasm IRS (0-12) mean.(range)	P –value (MW-U)	Total IGF1R (0-24), mean.(range)	P –value (MW-U)
<b>RwR (n=23)</b>	1.0(0-6)		2.17(0-6)		3.17(0-10)	
<b>Radiotherapy Failure</b>						
Primary Biopsy (n=7)	2.29(0-6)	0.2	3.14(0-8)	0.54	5.43(0-12)	0.4
Recurrent Biopsy (n=14)	3.07(0-9)	0.004	3.36(0-8)	0.18	6.43(2-15)	0.012
Overall Mean (n=18)	3.04(0-9)	0.0009	3.62(0-8)	0.08	6.66(0-14)	0.011
<b>Primary Laryngectomy (n=20)</b>	4.25(0-9)	0.0003	4.05(0-12)	0.022	8.30(0-17)	0.02

**Table 1-2:** Immunoreactive Scores IGF-1R - RwR vs. Radiotherapy Failure vs. Primary Laryngectomy  
(p values from MW-U test compared to RwR cohort).

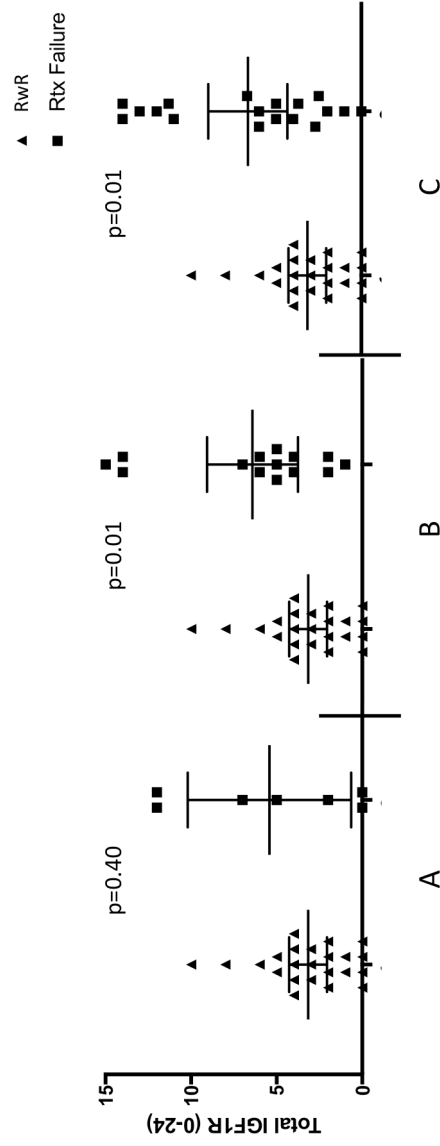
*Total and membrane IGF-1R scores were higher in patients with Rtx failure  
and Primary laryngectomy when compared to samples from patients with LTR.*

Abbreviations: MW-U, Mann-Whitney U Test; IRS, immunoreactive score; IGF-1R, insulin like growth factor receptor type 1; RwR, radiotherapy without known recurrence .

The membrane, cytoplasmic and total IGF-1R scores were higher in the radiotherapy failure group (2.29, 3.14, 5.43 vs. 1.00, 2.17, 3.17 respectively) although the differences were not statistically significant (membrane IGF-1R  $p=0.2$ , cytoplasmic IGF-1R  $p=0.54$ , total IGF-1R  $p=0.40$ ; **Figure 1-3a**).

Primary biopsies from those receiving RwR ( $n=23$ ) were then compared to biopsies taken following disease recurrence ( $n=14$ ) (see **Figure 1-1**). The membrane, cytoplasmic and total IGF-1R scores were higher in the radiotherapy failure group (respectively 3.07, 3.36, 6.43 vs. 1.0, 2.17, 3.17  $p=0.004$ ,  $p=0.18$ ,  $p=0.01$ ). The difference in membrane and total IGF-1R were statistically significant (**Figure 1-3b**).

An additional analysis was conducted comparing the mean IGF-1R IRS scores of all available specimens from patients with radiotherapy failure ( $n=18$ ) to those receiving RwR ( $n=23$ ) (**Figure 1-3c**). Membrane, cytoplasmic and total IGF-1R scores were higher in the radiotherapy failure group (3.04, 3.62, 6.66 vs. 1.0, 2.17, 3.17,  $p=0.009$ ,  $p=0.08$ ,  $p=0.01$ ). The results were significant when comparing membrane and total IGF-1R.



**Figure 1-3:** Total IGF-1R expression in RwR vs. radiotherapy failure: **A.** primary biopsies **B.** primary vs. recurrence **C.** primary vs. mean score all available biopsies from patients with Rtx failure. *Graphs show mean total IGF-1R score  $\pm$  95% confidence intervals.*

Abbreviations: Rtx, radiotherapy; IGF-1R, insulin like growth factor receptor type 1; RwR, radiotherapy without known recurrence

Similar comparisons were made between membrane, cytoplasmic and total EGFR scores between patients with RwR and radiotherapy failure (see **Table 1-3** and **Figure 1.4**). There was no statistically significant difference in EGFR expression between the RwR and radiotherapy failure groups.

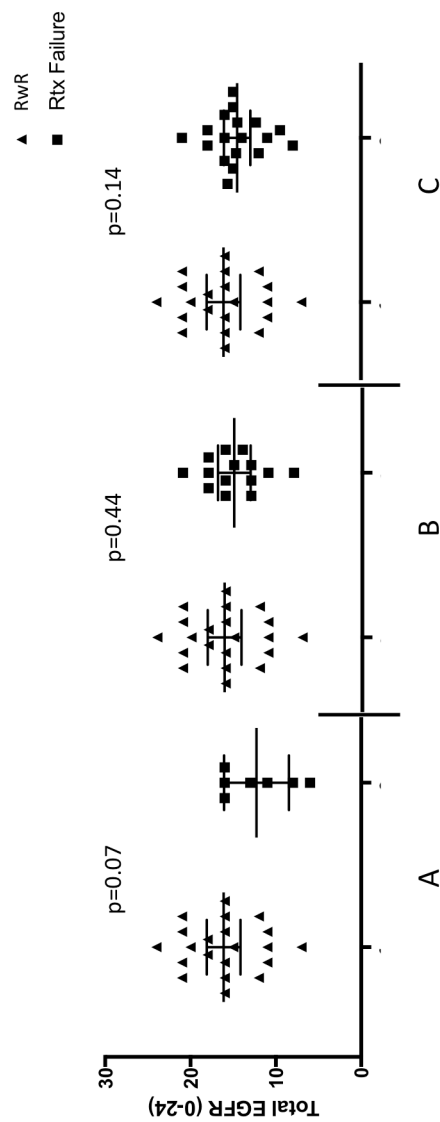


	Membrane IRS (0-12), mean.(range)	P-value (MW-U)	Cytoplasm IRS (0-12), mean.(range)	Total EGFR (0-24), mean.(range)	P-value (MW-U)
<b>RwR (n=21)</b>	10.33(4-12)		5.81(3-12)	16.14(7-24)	
<b>Radiotherapy Failure</b>					
Primary Biopsy (n=7)	9.43(4-12)	0.41	2.86(0-4)	12.29(6-16)	0.07
Recurrent Biopsy (n=14)	10.86(8-12)	0.66	4.14(0-9)	15(8-21)	0.44
Overall Mean (n=18)	10.31(6-12)	0.62	4.22(0-9)	14.54(8-21)	0.14
<b>Primary Laryngectomy(n=22)</b>	10.55(6-12)	0.83	5.77(2-12)	16.32(9-24)	0.9

**Table 1-3: Immunoreactive Scores EGFR - RwR vs. Radiotherapy Failure vs. Primary Laryngectomy.**

*EGFR scores were similar when comparing specimens from each cohort of patients.*

Abbreviations: MW-U, Mann-Whitney U test; IRS, immunoreactive score; EGFR, epidermal growth factor receptor.



**Figure 1-4:** Total EGFR expression in RwR vs. radiotherapy failure:

**A.** primary biopsies **B.** primary vs. recurrence **C.** primary vs. mean score all available biopsies from patients with Rx failure.

*Graphs show mean total EGFR score  $\pm$  95% confidence intervals.*

Abbreviations: Rx, radiotherapy; EGFR, epidermal growth factor receptor.

#### 1.4.2 Radiotherapy Failure: Primary vs. Recurrence

Pre and post radiotherapy membrane, cytoplasmic and total IGF-1R and EGFR scores were compared in the radiotherapy failure group to determine whether IGF-1R expression had altered after radiotherapy.

There were 6 patients with radiotherapy failure, from whom both pre and post radiotherapy tumour samples were available. A paired t-test analysis was conducted to assess IGF-1R and EGFR levels pre and post radiotherapy in these individuals (**Table 1-4a**, **Table 1-4b**, **Figure 1-5a** and **Figure 1-5b**). Membrane, cytoplasmic and total IGF-1R scores all showed a significant increase after radiotherapy (2, 2.3, 4.3 vs. 4.2, 4.3, 8.5  $p < 0.05$ ). Whilst membrane, cytoplasmic and total EGFR levels did rise after radiotherapy (9, 2.67, 11.67 vs. 11, 3.83, 14.83  $p > 0.05$ ), the increases were not statistically significant.

	Membrane IRS (0-12), mean.(range)	P –value (paired t-test)	Cytoplasm IRS (0-12), mean.(range)	P –value (paired t-test)	Total IGF1R (0-24), mean.(range)	P –value (paired t-test)
<b>Primary Biopsy (n=6)</b>	2 (0-9)		2.3(0-6)		4.3(0-12)	
<b>Recurrence (n=6)</b>	4.2(0-9)	0.048	4.3(2-8)	0.033	8.5(4-15)	0.006

**Table 1-4a:** Immunoreactive Scores – Radiotherapy Failures : Primary Biopsy vs. Recurrent Biopsy – IGF-1R

for samples where a primary and recurrence biopsy were available from the same patient.

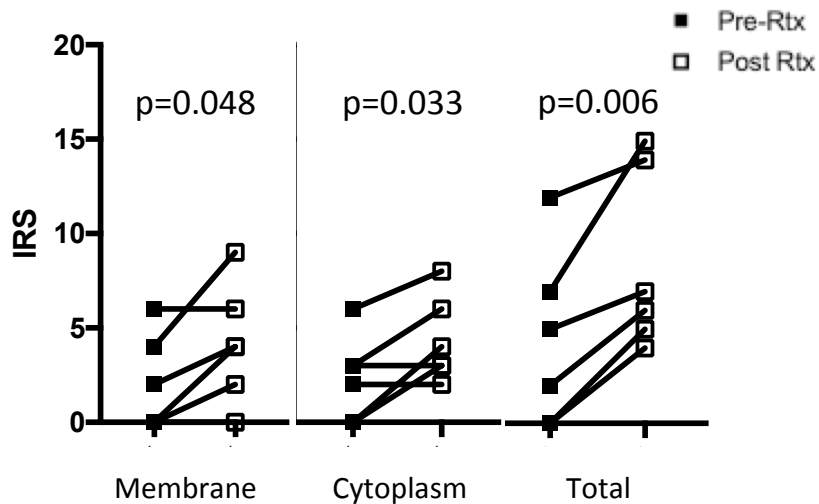
*Total, membrane and cytoplasmic IGF-1R levels were higher after radiotherapy.*

	Membrane IRS (0-12), mean.(range)	P –value (paired t-test)	Cytoplasm IRS (0-12), mean.(range)	P –value (paired t-test)	Total EGFR (0-24), mean.(range)	P –value (paired t-test)
<b>Primary Biopsy (n=6)</b>	9(4-12)		2.67(0-4)		11.67(6-16)	
<b>Recurrence (n=6)</b>	11(9-12)	0.08	3.83(1-6)	0.38	14.83(13-18)	0.13

**Table 1-4b:** Immunoreactive Scores – Radiotherapy Failures : Primary Biopsy vs. Recurrent Biopsy – EGFR

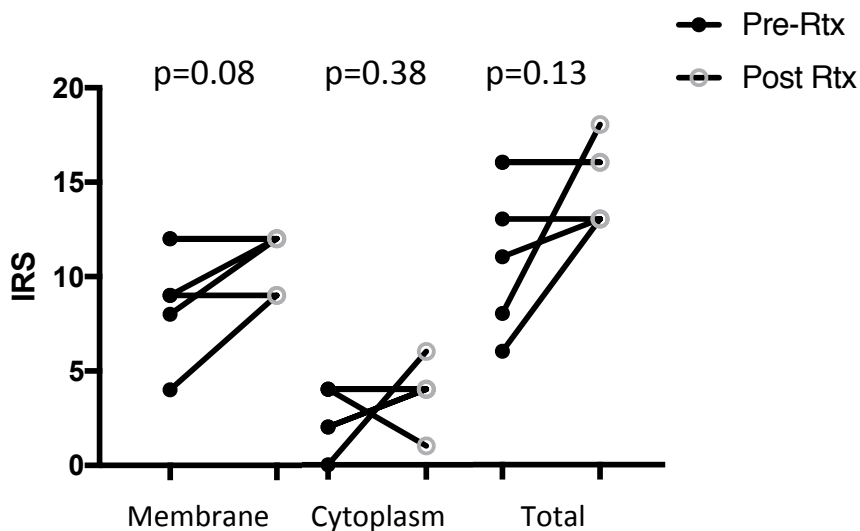
for samples where a primary and recurrence biopsy were available from the same patient.

*Whilst there was a trend towards increased EGFR after radiotherapy, the results were not statistically significant.*



**Figure 1-5a:** IRS for IGF-1R in Primary vs. Recurrent Biopsies in Paired Specimens from the Same Patient. *Graphs show paired membrane, cytoplasmic and total IGF-1R scores pre and post radiotherapy in patients with Rtx failure. The connecting line represents paired samples.*

Abbreviations: Rtx, radiotherapy; IRS, immunoreactive score.



**Figure 1-5b:** IRS for EGFR in Primary vs. Recurrent Biopsies in paired specimens from the same patient. *Graphs show paired membrane, cytoplasmic and total EGFR scores pre and post radiotherapy in patients with Rtx failure. The connecting line represents paired samples.*

Abbreviations: Rtx, radiotherapy; IRS, immunoreactive score.

#### 1.4.3 Primary vs. Salvage Total Laryngectomy

The membrane, cytoplasmic and total IGF-1R and EGFR levels were also compared between primary total laryngectomy and salvage laryngectomy to see if previous radiotherapy or advanced disease were associated with IGF-1R and EGFR expression (see **Table 1-5** and **Figure 1-6**). There was no significant difference between the two groups in terms of membrane, cytoplasmic and total IGF-1R ( $p=0.62$ ,  $p=0.78$ ,  $p=0.87$ ) and EGFR expression ( $p=0.52$ ,  $p=0.64$ ,  $p=0.43$ ).

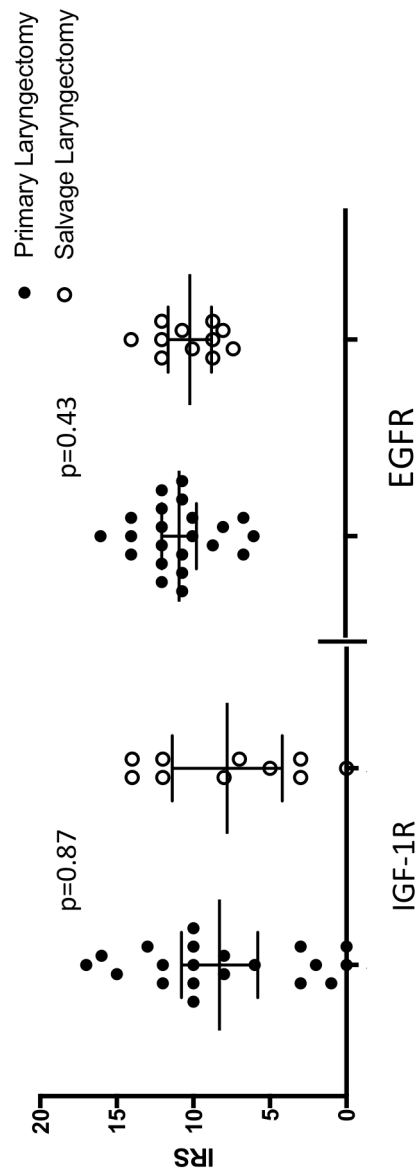
	Membrane IRS (0-12), mean.(range)	P –value (MW-U)	Cytoplasm IRS (0-12) mean.(range)	P –value (MW-U)	Total IGF1R (0-24), mean.(range)	P –value (MW-U)
<b>Primary Laryngectomy (n=20)</b>	4.25(0-9)		4.05(0-12)		8.30(0-17)	
<b>Salvage Laryngectomy (n=10)</b>	3.60(0-6)	0.62	4.20(0-8)	0.78	7.80(0-14)	0.87

	Membrane IRS (0-12), mean.(range)	P value (MW- U)	Cytoplasm IRS (0-12), mean.(range)	P –value (MW-U)	Total EGFR (0-24), mean.(range)	P –value (MW-U)
<b>Primary Laryngectomy (n=22)</b>	10.55(6-12)		5.77(2-12)		16.32(9-24)	
<b>Salvage Laryngectomy (n=11)</b>	10.09(6-12)	0.52	5.18(2-9)	0.64	15.27(11-21)	0.43

**Table 1-5:** Immunoreactive Scores for IGF-1R and EGFR in Primary vs. Salvage Total Laryngectomy.  
*IGF-1R and EGFR levels were similar in both cohorts.*

Abbreviations: MW-U, Mann-Whitney U test; IRS, immunoreactive score; EGFR, epidermal growth factor receptor; IGF-1R, insulin like growth factor receptor type 1.



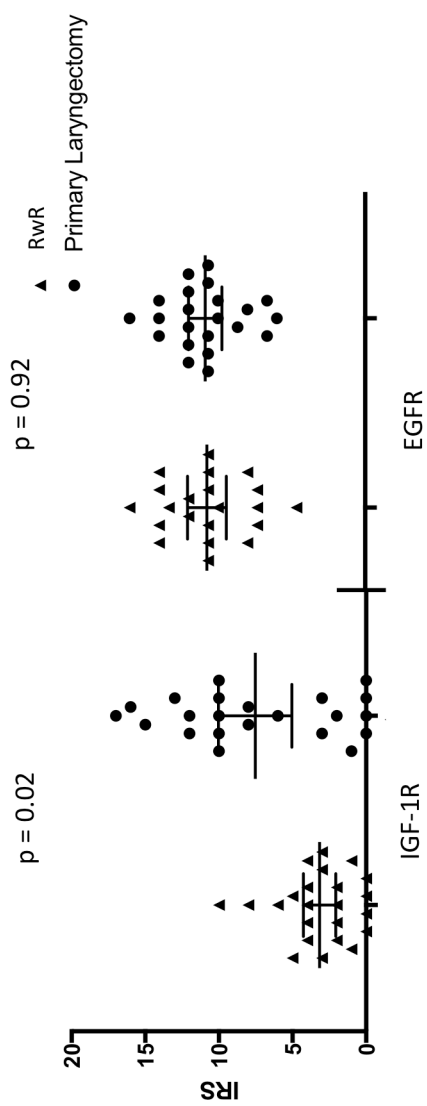
**Figure 1-6:** Total IGF-1R and EGFR in Primary vs. Salvage Total Laryngectomy  
*Graphs show mean total IGF-1R and EGFR scores  $\pm$  95% confidence intervals.*

Abbreviations: IRS, immunoreactive score; Rtx, radiotherapy; EGFR, epidermal growth factor receptor; IGF-1R, insulin like growth factor receptor type 1.



#### 1.4.4 Radiotherapy without known Recurrence vs. Primary Total Laryngectomy

Tumour samples from patients with RwR were compared to samples taken from primary total laryngectomies (see **Table 1-2, 1-3** and **Figure 1-7**). As expected patients undergoing primary total laryngectomy had a higher T and N stage ( $p < 0.05$ ). The membrane, cytoplasmic and total IGF-1R levels were significantly higher in the primary total laryngectomy group (4.25, 4.05, 8.30 vs. 1.00, 2.17, 3.17,  $p = 0.0003$ ,  $p = 0.02$ ,  $p = 0.02$ ) whilst there was no difference in EGFR scores (10.55, 5.77, 16.32 vs. 10.33, 5.81, 16.14,  $p = 0.83$ ,  $p = 0.63$ ,  $p = 0.9$ ).



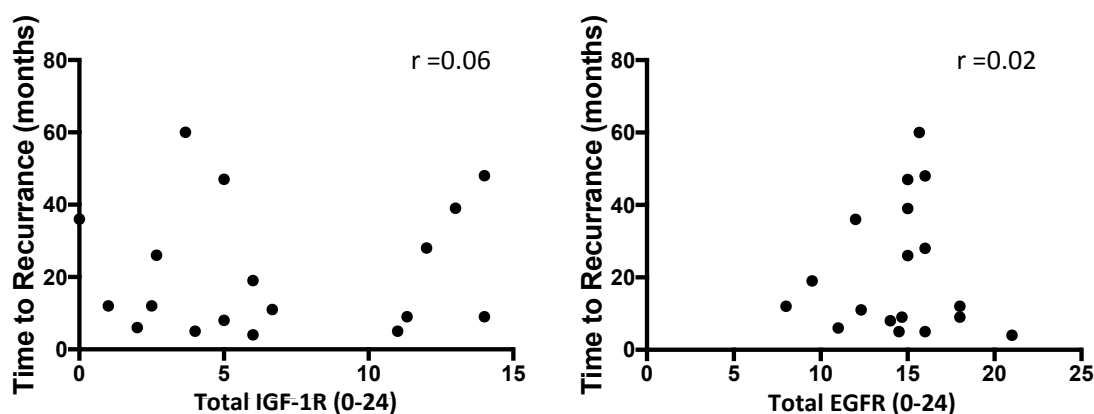
**Figure 1-7:** Total IGF1R and EGFR in RwR vs. Primary Total Laryngectomy.

*Graphs show mean total IGF-1R and EGFR scores  $\pm$  95% confidence intervals.*

Abbreviations: IRS, immunoreactive score; EGFR, epidermal growth factor receptor; IGF1R, insulin like growth factor receptor type 1; RwR, radiotherapy without known recurrence.

#### 1.4.5 IGF-1R Levels and Time to Recurrence

Membrane, cytoplasmic and total IGF-1R and EGFR scores were compared to the time taken for recurrence in the radiotherapy failure group to see if IGF-1R and EGFR levels were associated with earlier disease recurrence (see **Figure 1-8**). There was no correlation between IGF-1R or EGFR levels and time to disease recurrence.



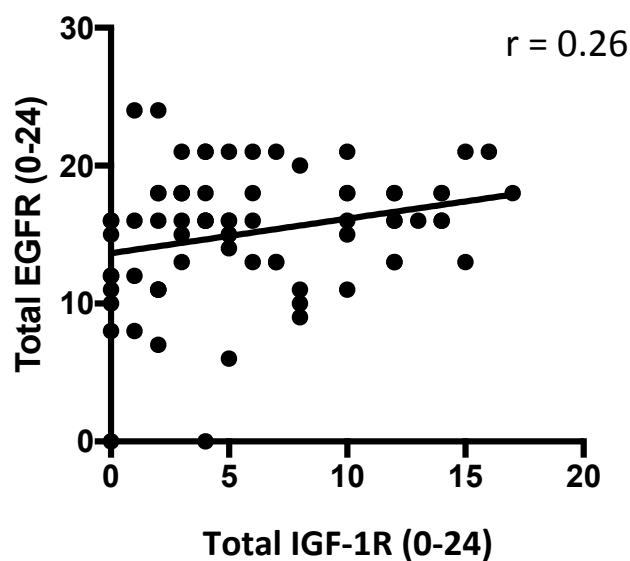
**Figure 1-8:** Total IGF-1R and EGFR vs. time to recurrence in Rtx failure group.

*Graphs show correlation between IGF-1R and EGFR total scores and time to recurrence in patients with Rtx failure.*

Abbreviations: Rtx, radiotherapy; r, Pearson's correlation coefficient; EGFR, epidermal growth factor receptor; IGF-1R, insulin like growth factor receptor type 1.

#### 1.4.6 Correlation Between IGF-1R and EGFR

Where both EGFR and IGF-1R expression was detected in the same sample, IRS scores were compared. There was no statistically significant correlation between membrane, cytoplasmic and total EGFR and IGF-1R in patients with RWR, radiotherapy failure or primary total laryngectomy alone. When the results for all samples were combined (see **Figure 1-9**), higher levels of EGFR were associated with elevated IGF-1R levels ( $r = 0.26$  ;0.03-0.45 95% confidence interval,  $p=0.02$ ).



**Figure 1-9:** Correlation between Total IGF1R and Total EGFR in all samples where IGF-1R and EGFR IHC was performed.

*The line of best fit is displayed and demonstrates a correlation between IGF-1R and EGFR levels.*

Abbreviations: r, Pearson's correlation coefficient; EGFR, epidermal growth factor receptor; IGF-1R, insulin like growth factor receptor type 1.

### **1.5 Discussion:**

Radiotherapy failure in the context of LSCC can have disastrous consequences on patients both in terms of radiotherapy related morbidity and complications of salvage surgery. Given the rising proportion of patients receiving primary radiotherapy for LSCC and static overall survival [27] there is a clinical need to identify biomarkers of radiotherapy resistance in this group of patients. This study was designed to determine if levels of IGF-1R expression were able to predict radiotherapy resistance in individuals with LSCC.

This study compared patients experiencing RwR vs. recurrence following radiotherapy for LSCC. The groups were similar both in terms of tumour size and patient factors. IGF-1R IHC in the primary biopsies showed a trend to higher pre-treatment IGF-1R levels in patients experiencing radiotherapy failure, although the differences were not statistically significant. This may have been due to relatively low sample numbers (n=7) in the radiotherapy failure group. EGFR levels were similar when comparing patients experiencing RwR vs. radiotherapy failure.

This conflicts with previously published evidence that higher levels of EGFR associate with radiotherapy resistance in HNSCC [60,61]. Demiral et al (2004) [61] found higher levels of EGFR associated with radiotherapy resistance in early glottic cancer. In the present larger study, all tumour specimens were found to express high levels of membrane EGFR, this may have reflected the cohort of patients selected, IHC or scoring method utilised.

To determine if IGF-1R expression was elevated post radiotherapy we compared IGF-1R levels from patients with radiotherapy failure prior to and following radiotherapy. A paired analysis of tumour samples taken pre and post radiotherapy demonstrated a significant increase in membrane, cytoplasmic and total IGF-1R post irradiation. These results suggested a potential association between elevated IGF-1R and radiotherapy resistance and rising IGF-1R levels following radiotherapy in LSCC.

The IGF-1R is a receptor tyrosine kinase known to have a role in cell cycle progression, proliferation, differentiation, survival, malignant transformation, tumour invasion, metastasis and inhibition of cellular apoptosis [62]. Radiotherapy induces DNA damage through nucleotide excision, single and double stranded breaks, the latter being the most significant for cellular death [63]. In some cases cells are able to repair DNA damage; if this does not take place the process of apoptosis begins [63]. There are two primary mechanisms for cellular repair; homologous recombination (HR) and non homologous end joining (NHEJ) [64]. NHEJ is present throughout the cell cycle and is the most extensive repair pathway for double stranded break DNA repair in mammals [65]. During NHEJ, damaged bases are resected and the ends re-joined resulting in a loss of information within damaged cells. This process introduces genomic instability and may favour survival amongst altered phenotypes. IGF-1R overexpression is thought to help mediate this mechanism, thereby favouring malignant transformation alongside resistance to radiotherapy [63,66], it might also be up regulated after irradiation as seen in the present study.

Following radiotherapy non-cancer cells typically undergo apoptosis, however cancer cells particularly those resistant to radiotherapy can behave very differently, and up

regulation of IGF-1R after radiotherapy could be the result of an altered response to irradiation and DNA repair. Dysregulation of the 'normal' cell cycle and resistance to programmed cell death are some of the hallmarks of cancer [67]. Radiotherapy induces DNA damage, which if left unrepaired typically results in programmed cell death. Malignant cells however, are often resistant to the normal molecular mechanisms for cell cycle regulation, programmed cell death and DNA repair e.g. p53 mutation and NHEJ [68]. They exhibit increased cellular proliferation, altered DNA repair and resistance to apoptosis, IGF-1R is involved in each of these cellular processes [63].

IGF-1R signalling along the MAPK pathway results in increased cellular proliferation [69]. IGF-1R also associates with the function of ATM kinase (a protein critical for cell cycle progression by regulating cell proliferation and inhibiting apoptosis along the PI3K pathway) [70]. ATM kinase is usually activated in response to ionising radiation resulting in DNA repair, apoptosis and cell cycle arrest, however up regulation of IGF-1R is known to inhibit this effect and can result in altered DNA repair [71]. The present study demonstrated up regulation of IGF-1R after radiotherapy in patients with radiotherapy failure, this could be a feature of tumours with an innate resistance to radiotherapy due to elevated IGF-R. It might also reflect the selective survival of tumour cells within heterogenous tumours exhibiting higher IGF-1R levels. It might also be a direct consequence of altered DNA repair after radiotherapy promoting the survival of cells expressing elevated IGF-1R given the survival advantage it provides malignant cells. The true mechanism is likely to be multifactorial and requires further characterisation and investigation.

There are multiple studies over the past 2 decades evaluating the prognostic value and relative radiotherapy resistance due to IGF-1R overexpression in other solid organ tumours. In vitro, studies suggest that IGF-1R expressing mouse embryo fibroblasts, murine melanoma cells and human breast cancer cells can be radio-sensitised by targeting IGF-1R [72-74]. Our study design and results were similar to the first clinical study looking at IGF-1R as a potential marker of radiotherapy resistance. Turner et al (1997) [73] compared tumour tissue taken from breast cancer patients following early local relapse after radiotherapy to those without relapse and found significantly higher IGF-1R levels in the radiotherapy failure group. Following their work there have been similar studies demonstrating elevated levels of IGF-1R in radiotherapy resistant cervical cancer [53,75] and an association between IGF-1R overexpression and survival in colorectal [76] and non-small cell lung cancer [77]. A single study has contradicted these findings; Peiro et al (2009) [78] reported better treatment response for patients with high levels of IGF-1R treated with lumpectomy and radiotherapy for breast cancer. Differences between the IHC and scoring methods used by these authors and in the literature and a lack of data relating to patient selection has made interpretation of these results in the context of other studies difficult.

The relationship between IGF-1R expression and HNSCC has previously been investigated. Studies of nasopharyngeal [79] and oral cavity cancers [80] have shown poorer outcomes from radiotherapy for patients with higher levels of IGF-1R. Previous work from our group has also highlighted an association between high levels of IGF-1R and T-stage, HPV negative status and adverse survival in HNSCC [54]. The results of the present study support these findings, as we found higher levels of IGF-1R in samples taken from primary and salvage laryngectomies when compared to patients



receiving primary radiotherapy, suggesting a positive association between IGF-1R expression and larger tumour or T-stage.

Given these results, IGF-1R levels might represent a surrogate marker of T-stage. Studies have previously shown an association between IGF-1, IGF-1R levels and tumour growth, depth of invasion and lymphatic and venous invasion in colorectal, lung and head and neck cancer [81,82,54]. As IGF-1 is one of the growth factors associated with normal cellular growth [83], and deletion of IGF-1R causes a 50% reduction in the size of mouse embryos [84] and drosophila [85] this might explain the correlation between IGF-1R expression and increased tumour size. The increase in tumour size associated with IGF-1R levels might also explain the association of elevated IGF-1R with decreased overall survival for a number of solid organ tumours including head and neck cancer and lung cancer [86,54].

Matsumoto et al (2016) [55] assessed IGF-1R levels using IHC in patients with early stage LSCC treated with primary radiotherapy. In their study of diagnostic biopsies from 43 patients, local recurrence was experienced by 9 of 25 patients with high IGF-1R and 1 of 18 of those with low tumour IGF-1R. They found no association between IGF-1R levels and time to recurrence, as did our study.

However, the association of high IGF-1R with an increased likelihood of radiotherapy failure, was not absolute, as Matsumoto et al (2016) [55] reported treatment failure in one patient with low IGF-1R and successful treatment in 16 patients with high IGF-1R levels. Despite their findings, they concluded primary radiotherapy be utilised as first line treatment for early glottic cancer irrespective of any predictive factors.

These conclusions are based on the presumption that salvage surgery remains a viable curative option for patients with radiotherapy failure, and that radiotherapy provides superior functional outcomes in comparison to primary surgery. Whilst these conclusions are logical, there is sufficient evidence to suggest at least equivalent functional outcomes for patients treated with radiotherapy and surgery in laryngeal cancer. With regards to voice, Taylor et al (2013) [87] reported equivalent functional outcomes for patients with early laryngeal cancer treated with primary trans oral microsurgery and radiotherapy. In fact, a recent meta-analysis found superior voice outcomes for patients treated with laser surgery when compared to radiotherapy [88]. Burnip et al (2013) [89] reported similar swallowing outcomes for patients undergoing radiotherapy or surgery in advanced laryngeal cancer. Whilst the retrospective nature of these studies is a limiting factor their conclusions should not be ignored. An alternative conclusion that Matsumoto et al (2016) [55] may have considered, would have been to recommend the most effective primary treatment based on tumour predictors. This might be radiotherapy or surgery with salvage surgery or radiotherapy held in reserve in the case of treatment failure. This approach might limit patient morbidity by utilising single modality treatment and potentially improve overall survival.

The present study included a larger number of patients with both early and advanced stage tumours and assessed EGFR in addition to IGF-1R when compared to Matsumoto et al (2016) [55]. Given the recent description of EGFR as one of the hallmarks of head and neck cancer [90] and the potential for cross communication with IGF-1R via the P13K/AKT pathway [91] we felt it was important to evaluate both receptors simultaneously. Interestingly, our study found a weakly positive association between

EGFR and IGF-1R expression, further adding to the evidence behind a biological association between these two receptor tyrosine kinases [51,92,91].

Our IHC score for IGF-1R included assessment of both intensity and proportion of tumour stained to determine an IRS, whilst Matsumoto et al [55] assessed only proportion of tumour stained to determine IGF-1R expression. Using this single stage method relatively low intensity widespread staining could be interpreted as high expression whilst this would not occur with the two-stage method used in our study. It is possible, therefore, that Matsumoto et al [55] may have overestimated the proportion of patients with high IGF-1R and conversely underestimated the prognostic value of IGF-1R in detecting radiotherapy failure.

Other strengths of our study included the use of blinding to limit bias during IHC interpretation, the use of adjacent tumour sections when comparing EGFR and IGF-1R and control samples for each IHC staining run. Our study was limited by its retrospective nature, low numbers of diagnostic samples in the radiotherapy failure group, missing samples and the generic limitations of IHC interpretation. Whilst two scorers were used to overcome subjectivity and conflicts were resolved by consensus interpretation using a microscope with a teaching arm, the nature of IHC meant that only a representative portion of the tumour was analysed. Although whole mount sections are superior to tissue microarrays in large or particularly heterogeneous tumours they may not represent the true tumour profile. A variation in the total length of follow up for patients with RwR (range 1-5yrs) might also have influenced the outcomes of comparisons.

The present study suggests that IGF-1R is up regulated following radiotherapy for LSCC, and provides support from previous investigations into IGF-1R [54,60,79,80] for an association between elevated IGF-1R and radiotherapy resistance in LSCC and other types of HNSCC.

So far only human papilloma virus (HPV) has shown promise as a biomarker for treatment decisions in oropharyngeal squamous cell carcinoma (OPSCC) [93]. There are no clinically utilised biomarkers for personalised therapy in LSCC. If our results are replicated in large-scale prospective studies, IGF-1R status could help refine current treatment decisions. Much like HPV testing for OPSCC, IGF-1R testing could contribute to treatment decisions in LSCC. Patients with high IGF-1R could be offered primary surgery with radiotherapy and chemotherapy held in reserve. This could alter current trends towards primary radiotherapy based on TNM status and patient factors [30] to treatment guided by the molecular profile or IGF-1R status of tumours. Before implementing these changes it will be necessary to determine the clinically relevant definition of 'IGF-1R overexpression' given the lack of a current consensus regarding this.

Personalised medicine has become a reality in the management of many solid organ tumours including breast, ovarian, prostate, colorectal, skin and non-small cell lung cancer[4]. Treatments for HNSCC however have not kept pace; testing for IGF-1R status or molecular therapies aimed at targeting this receptor [94,95] may help to overcome the current limitations with radiotherapy resistance in LSCC and improve overall survival.

## **1.6 Conclusions:**

This study found a trend to higher IGF-1R in primary biopsies of patients with LSCC experiencing radiotherapy failure, with evidence of significant IGF-1R up regulation following radiotherapy. IGF-1R levels were also positively associated with increased tumour size and weakly associated with EGFR expression. This could mean that IGF-1R expression might also represent a surrogate measure of T stage. There was also a weak association between radiotherapy and up regulation of EGFR, although this was not statistically significant.

Whilst these results should be interpreted cautiously given the retrospective design, missing data and relatively small numbers, it is the largest study of its type and the results warrant further investigation. The outcomes from on-going trials of IGF-1R inhibition using monoclonal antibodies independently and in combination with EGFR monoclonal antibody Cetuximab [96-98] may provide further prognostic information for the role of IGF-1R in radiotherapy resistant HNSCC. Prospective large-scale multi-centre trials are needed to test these findings and determine the ‘clinically relevant’ level to define IGF-1R overexpression to guide current and new emerging treatments.

## **Chapter 2: Investigating Insulin-Like Growth Factor Receptor 1 (IGF-1R) as a Potential Driver of Radiation Resistance in Laryngeal Cancer.**

### **Abstract:**

#### **Background:**

There is a clinical need for predictive biomarkers of radiotherapy resistance in laryngeal cancer (LSCC). At present we are unable to predict the individuals likely to benefit from radiotherapy and which are likely to fail. The drivers of radiotherapy resistance in LSCC are unclear, but both the epidermal growth factor receptor (EGFR) and insulin like growth factor receptor type 1 (IGF-1R) have been implicated.

#### **Aims:**

To develop an in vitro model of radiation resistance in laryngeal cancer cells, characterise EGFR and IGF-1R expression and activation, and investigate the effects of EGFR and IGF-1R inhibition on radio-sensitivity.

#### **Methods:**

Two laryngeal cancer cell lines (BICR18 and SQ20B) were repeatedly irradiated using clinically utilised radiotherapy treatment schedules to a total of 55 or 60 Gray (Gy). Western blots were used to compare IGF-1R, EGFR and downstream target expression and activation between the parental and irradiated cell lines. Clonogenic survival assays (CSAs) were used to assess relative radiotherapy resistance. Cells were treated with BI836845 (IGF ligand antibody) and Afatinib (EGFR receptor tyrosine kinase inhibitor) alone and in combination to determine the effects on response to radiation. Drug efficacy and radiation response was tested using western blots and CSA.

**Results:**

SQ20B cells expressed IGF-1R and EGFR, BICR18 cells expressed IGF-1R with no detectable EGFR. In SQ20B cells there was evidence of receptor cross talk, whereby EGF ligand was shown to activate IGF-1R. SQ20B cells irradiated to a dose of 55Gy were more radio-resistant than parental cells. Repeated irradiation of BICR18 cells did not produce a radiotherapy resistant phenotype. Both irradiated cell lines demonstrated increased IGF-1R expression and EGFR was increased in irradiated SQ20B cells. EGFR inhibition was found to radio sensitize SQ20B cells, but combined EGFR/IGF-1R inhibition was no more effective. However, assays of basal cell survival (in absence of radiotherapy) in SQ20B cells showed that combined EGFR/IGF-1R inhibition was more effective, than either single agent, this effect was greater in previously irradiated cells.

**Conclusions:**

IGF-1R was up regulated following repeated irradiation in LSCC cell lines, but did not appear to be a driver of radiotherapy resistance. Combined IGF-1R and EGFR inhibition reduced cell survival in the absence of radiation in repeatedly irradiated SQ20B cells, suggesting induced dependence on these pathways. Further work is needed to understand the interaction between EGFR and IGF-1R in radiotherapy resistant LSCC.

## 2.1 Background:

T1-T2 laryngeal squamous cell carcinoma (LSCC) is frequently treated with radiotherapy[34]. Some centres have reported 5-year survival at 94% [99] whilst others have reported local or regional recurrence in up to 21% and 37% of T1 and T2 glottic LSCCs respectively [40,41]. The mechanisms for radiotherapy resistance in LSCC are unclear, but both the epidermal growth factor receptor (EGFR) [90] and insulin like growth factor receptor 1 (IGF-1R) have been implicated[54].

Clarification of the role for these two receptor tyrosine kinases (RTKs) and their interaction could aid prediction of radiotherapy resistance in LSCC. The presence and abundance of both IGF-1R and EGFR may represent important biomarkers for predicting treatment response in LSCC.

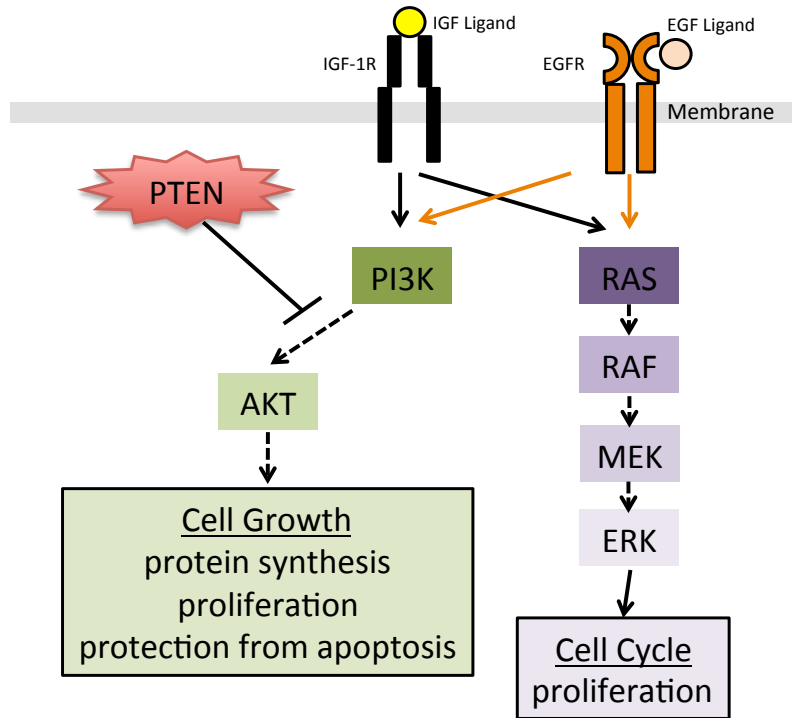
### EGFR and IGF-1R in Laryngeal Cancer

EGFR is a receptor tyrosine kinase (RTK) present in the plasma membrane and cell nucleus and is a promising therapeutic target in head and neck cancer (HNSCC) [13]. EGFR overexpression is considered one of the ‘hallmarks of HNSCC’ [90]. It is overexpressed in more than 80% of HNSCCs [48,49] and is present in the early stages of tumor genesis, marking it as a potential driver of tumour formation. EGFR levels are typically 1.7x greater in HNSCC tissue than in the squamous epithelium of non-smokers without HNSCC [100]. EGFR overexpression is also seen in the normal epithelium of HNSCC patients, and may contribute to the 36% risk of a second primary tumour [101]. As mucosa transitions from premalignant to malignant disease, EGFR expression is known to increase, and is associated with a poorer prognosis, increased loco-regional recurrence and increased resistance to radiotherapy [14,15].



In vitro, Cetuximab a monoclonal antibody to EGFR causes HNSCC cells to accumulate in the G1 phase of the cell cycle, inhibiting proliferation and increasing radiotherapy sensitivity [102]. Randomized controlled trials (RCT) of radiotherapy vs. radiotherapy and Cetuximab in HNSCC have shown loco-regional control and 5-year survival of 14.9 months and 36.4% vs. 24.4 months and 45.6% respectively [17,18]. On this basis, Cetuximab is now licensed for use in patients unsuitable for treatment with Cisplatin. However, we are still no closer to knowing which patients are most likely to benefit from this treatment [103].

An important issue with Cetuximab and other agents targeting EGFR is the development of treatment resistance [104]. The mechanisms for this remain unclear; receptor cross talk may have a role. EGFR and IGF-1R both communicate through the P13K/AKT [91] and RAS [105,106] signalling pathways. AKT and ERK are communal downstream targets for both receptors. The PI3K/AKT pathway has been implicated in cell growth and contributes to protein synthesis, cell proliferation and protection from apoptosis [92,91]. The tumour suppressor PTEN is known to regulate this pathway, loss of its activity can result in hyperactive signalling along this pathway [107]. The RAS pathway contributes towards regulation of the cell cycle and cellular proliferation (see **Figure 2-1a**) [92,91].



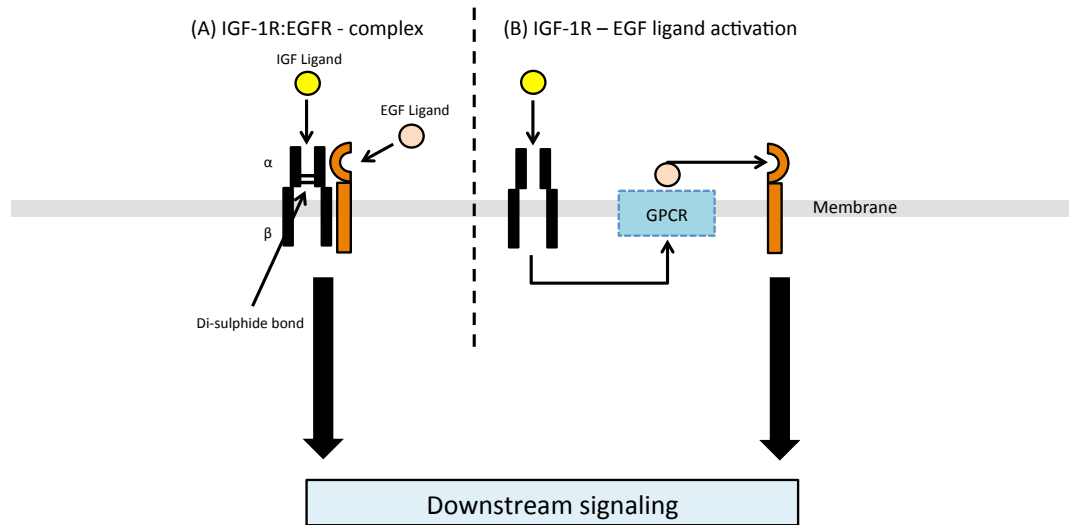
**Figure 2-1a:** Signalling pathway for IGF-1R and EGFR [92,91]

Abbreviations: IGF, insulin growth factor; IGF-1R, insulin like growth factor receptor type 1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPCR, G-protein coupled receptor.

*This figure demonstrates the activation of IGF-1R and EGFR by their respective ligand molecules and subsequent downstream signalling. Both receptors are known to communicate via the PI3K and RAS signalling pathways. These pathways are involved in cellular growth and proliferation respectively. Drugs targeting one of these receptors may prove ineffective, as compensatory downstream signalling might take place due to the other receptor e.g. IGF-1R compensates for loss of EGFR or vice-versa.*

The P13K/AKT and RAS signalling cascades are frequently mutated in cancer [108,109]; treatments targeting these pathways can result in anti-proliferative, anti-invasive and anti-angiogenic effects in HNSCC. It is plausible that dual inhibition of IGF-1R, EGFR and their downstream effectors could overcome resistance and improve treatment efficacy [51].

Receptor crosstalk is an important area of interest, and can occur when inhibition of one RTK e.g. EGFR may be overcome by activation of another e.g. IGF-1R [19,110]. This could happen through communal downstream signalling proteins or interaction at the cellular surface, several mechanisms have been proposed. These include the formation of IGF-1R/EGFR trans membrane complexes or through co-stimulation via membranous G-protein coupled receptors (see **Figure 2-1b**) [51].



**Figure 2-1b:** Proposed mechanism for ‘cross-talk’ between IGF-1R and EGFR (figure derived from van de Veen et al 2009 [51])

Abbreviations: IGF, insulin growth factor; IGF-1R, insulin like growth factor receptor type 1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; GPCR, G-protein coupled receptor.

(A) This figure illustrates a single IGF-1R molecule, with its extracellular  $\alpha$  and trans membrane  $\beta$  component forming a complex with a single EGFR molecule. It is proposed that both IGF and EGF ligand have the potential to stimulate downstream signalling of both receptors. (B) This figure illustrates another potential mechanism for ‘cross-talk’ between IGF-1R and EGFR. Here, it is thought that IGF ligand results in a stimulus to trans membrane G-protein coupled receptors resulting in the release of extracellular EGF ligand, thereby resulting in activation and downstream signalling for the EGFR receptor.

In previous work from our group, Dale et al [54] studied the expression of IGF-1R in HNSCC. They found that IGF-1R was overexpressed in HNSCCs, and high levels of IGF-1R associated with HPV negative HNSCC, increased tumour T stage and adverse survival. Other components of the insulin growth factor (IGF) axis are also implicated in the pathogenesis of HNSCC. High levels of IGFBP-3 are associated with shorter time to progression [13], whilst high levels of serum IGF-1 are associated with increased risk of second primary tumours [111].

Further investigation is needed to determine the relationship between IGF-1R and EGFR pathways in radiotherapy resistant LSCC.

## **2.2 Aims:**

1. To develop an in vitro model of radiation resistance in laryngeal cancer cells.
2. Determine the relative expression and activation of EGFR, IGF-1R and communal downstream targets.
3. To investigate the effects of EGFR and IGF-1R inhibition independently and in combination on cell survival and radio resistance in parental and repeatedly irradiated cells.

## **2.3 Methods:**

### **2.3.1 Cell Culture**

The laryngeal cancer cell lines SQ20B and BICR18 were obtained from Dr Geoff Higgins, Dept of Oncology, University of Oxford and European Collection of Cell Cultures (ECACC) respectively. Mycoplasma testing was negative (MycoAlert, Lonza Rockland Inc, Rockland, United States). Both cell lines were tested by short tandem

repeat (STR) genotyping. Short tandem repeats are multiple copies of a short identical DNA sequence found in direct succession on particular regions of a chromosome. STR allows for cell line specific DNA profiling by looking for a known 'DNA fingerprint', this is compared to cell specific data held in a public database to confirm the identify of cells[112]. STR confirmed the identify of BICR18 however validation was not possible for SQ20B due to a lack of public records.

The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal calf serum (Gibco) and 1% penicillin/streptomycin (Gibco). The medium for BICR18 also contained 2mM Glutamine (Gibco) and 0.4 micrograms/ml hydrocortisone (Sigma-Aldrich) and for SQ20B 1% non-essential amino acids (Gibco). Both cell lines were maintained in a humidified incubator at 37°C, 5% CO<sub>2</sub>. All cell culture was performed in a laminar flow cabinet.

### 2.3.2 Exposure to Ionising Radiation

SQ20B and BICR18 cells were plated at a density of  $1 \times 10^6$  in T75 flasks and allowed to reach 80-90% confluence. Cells were treated using two clinically utilised radiotherapy regimes for laryngeal cancer; 55 Gray (Gy) in 20 fractions (Fr) and 60 Gy in 30 Fr. Irradiation was carried out using a Caesium-137 irradiator (GSR D1, gamma Service) giving a dose of 2.75Gy or 2Gy daily to the cells over 4 and 6 weeks with a two-day gap at the weekend to allow recovery. Cells were passaged weekly at 90% confluence. Cell lines were allowed 14 days recovery after completing treatment, prior to further characterisation.

Early and late passages of both parental cell lines were maintained as controls. This meant that for each cell line there were 4 subtypes available for analysis. These were early passage parental cells (used as a control), late passage parental cells (mirroring the number of passages irradiated cells had undertaken), cells treated with 55Gy in 20Fr and 60Gy in 30Fr. There were a total of eight cell lines produced, named according to the treatment received, early passage BICR18, BICR18, 55GyBICR18, 60GyBICR18, early passage SQ20B, SQ20B, 55GySQ20B and 60GySQ20B.

### 2.3.3 Clonogenic Survival Assays (CSA)

#### *Comparing Relative Radiotherapy Resistance:*

For each cell line 2000 cells (unless otherwise specified) were counted using an automated cell counter (Invitrogen Countess, Thermo Fisher Scientific, Loughborough, UK) and seeded into triplicate 100mm cell culture dishes and allowed to attach overnight. Each dish was irradiated with 0 to 10 Gy, after 7 days the medium was removed, cells washed with phosphate buffered saline (PBS) and colonies fixed and stained with 50% methanol, 7% acetic acid and 0.1% Coomassie Blue.

#### *Effects of Drug Inhibition:*

To assess the effects of EGFR and IGF-1R inhibition on parental and irradiated laryngeal cancer cells, Afatinib (EGFR RTK inhibitor) and BI836845 (IGF ligand antibody) were obtained from Selleckchem (Munich, Germany) and Boehringer Ingelheim (Vienna, Austria) respectively.

After confirmation of target inhibition using western blot (see below), 2000 cells were counted and seeded into 100mm cell culture dishes. The following day, cells were treated with both drugs at 100nM and 1000nM independently and in combination for 24 hours before irradiation, untreated cells were used as controls. The drugs were removed and culture medium changed for drug free culture medium 24 hours after irradiation, and a further 7 days were allowed before colony counting.

#### *Colony Counting:*

Colonies with 50 or more cells were counted as survivors using an automated colony counter (GelCount, Oxford Optronics). Each assay was performed using triplicate technical replicates and at least three independent experimental replicates were performed (unless otherwise specified).

#### 2.3.4 Western Blotting

The cells of interest were cultured to 80-90% confluence in 100mm culture dishes and treated with human recombinant IGF-1 ligand (50nM) (Sigma-Aldrich, St Louis, USA) and/or human recombinant EGF ligand (20nM) (Sigma-Aldrich, St Louis, USA) for 20 minutes. In some cases, cells were also treated with Afatinib or BI836845 for 60 minutes, before addition of IGF-1 and/or EGF ligand for 20 minutes. Control cells were treated with dimethyl sulfoxide (DMSO) (solvent for Afatinib) to match the concentration present for each drug tested.

Culture medium was removed and cells washed with ice-cold phosphate buffered saline (PBS). Cells were scraped into 1ml of ice-cold PBS, transferred to eppendorf tubes and centrifuged at 13,500rpm for 30 seconds at 4°C. The supernatant was removed and cell



pellets were snap frozen on dry ice and ethanol. The pellets were immediately used for western blotting.

Cell pellets were then re-suspended in radio-immunoprecipitation assay (RIPA) lysis buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Triton, 150mM NaCl, 1mM EDTA and 20mM Tris pH7.5, supplemented with protease inhibitor cocktail (Roche©, West Sussex, UK), 1.5mM Pefabloc SC plus (Roche©, West Sussex, UK) and phosphatase inhibitor complex II and III (Sigma-Aldrich, St Louis, USA)). The lysates were incubated on ice for 30 minutes before centrifuging at 13,500rpm at 4°C for 15 minutes. The lysates were used for protein assay (Pierce<sup>TM</sup> BCA Protein Assay Kit, ThermoFisher Scientific, Loughborough, UK) to determine the volume required for 30-40 µg of protein. Equivalent amounts of protein were mixed with Laemelli sample buffer (70mM Tris pH6.8, 5% β-mercaptoethanol, 40% glycerol, 3% SDS and 0.05% bromophenol blue), denatured at 95°C for 3-5 minutes and loaded onto 8% polyacrylamide gels for SDS polyacrylamide gel electrophoresis (SDS-PAGE). Protein transfer to a nitrocellulose membrane (Hybond C extra, Amersham Biosciences) was achieved using TransBlot® Turbo Transfer System with TransBlot® Turbo Transfer buffer solution (Bio-Rad, Hertfordshire, UK).

Membranes were blocked in 5% non-fat dairy milk for 1 hour at room temperature, and primary antibody applied for 60 minutes at room temperature. A summary of the primary and secondary antibodies used including solvents and concentrations is provided in **Table 2-1**. Following three 5 minute washes with Tris-Buffered Saline and 0.1% Tween (TBS-T), bound antibody was detected by incubating with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (DAKO,

Ely, UK) in blocking buffer at room temperature for 30 minutes. Three further washes with TBS-T were performed and proteins detected with Enhanced Chemiluminescence (ECLplus, Amersham Pharmacia Biotech), and visualized on X-ray film.

Primary Antibody	Product code	Phosphor tyrosine targeted	Manufacturer	Concentration	Solvent
pEGFR	2236	Tyr 1068	Cell Signalling Technology ®	1:500	5% BSA
EGFR	2232	n/a	Cell Signalling Technology ®	1:1000	5% SM
pIGF-1R	3024	Tyr 1135/1136 Tyr 1150/1151	Cell Signalling Technology ®	1:500	5% BSA
IGF-1R	3027	n/a	Cell Signalling Technology ®	1:1000	5% SM
pAKT	4060	n/a	Cell Signalling Technology ®	1:500	5% BSA
AKT	9272	n/a	Cell Signalling Technology ®	1:1000	5% BSA
pERK	9101	n/a	Cell Signalling Technology ®	1:1000	5% BSA
ERK	4695	n/a	Cell Signalling Technology ®	1:2000	5% BSA
β-tubulin	T4026	n/a	Sigma Aldrich	1:2000	5% SM
PTEN	9556	n/a	Cell Signalling Technology ®	1:2000	5% BSA

Secondary Antibody	Product code	Manufacturer	Species	Concentration	Solvent
pEGFR	7076	Cell Signalling Technology ®	Mouse	1:10,000	5% SM
EGFR	7074	Cell Signalling Technology ®	Rabbit	1:5000	5% SM
pIGF-1R	7074	Cell Signalling Technology ®	Rabbit	1:2000	5% SM
IGF-1R	7074	Cell Signalling Technology ®	Rabbit	1:5000	5% SM
pAKT	7074	Cell Signalling Technology ®	Rabbit	1:5000	5% SM
AKT	7074	Cell Signalling Technology ®	Rabbit	1:5000	5% SM
pERK	7074	Cell Signalling Technology ®	Rabbit	1:5000	5% SM
ERK	7074	Cell Signalling Technology ®	Rabbit	1:5000	5% SM
β-tubulin	7076	Cell Signalling Technology ®	Mouse	1:5000	5% SM
PTEN	7076	Cell Signalling Technology ®	Mouse	1:5000	5% SM

**Table 2-1:** List of primary and secondary antibodies utilised for Western Blotting

Abbreviations: BSA, Bovine Serum Albumin; SM, skimmed milk.

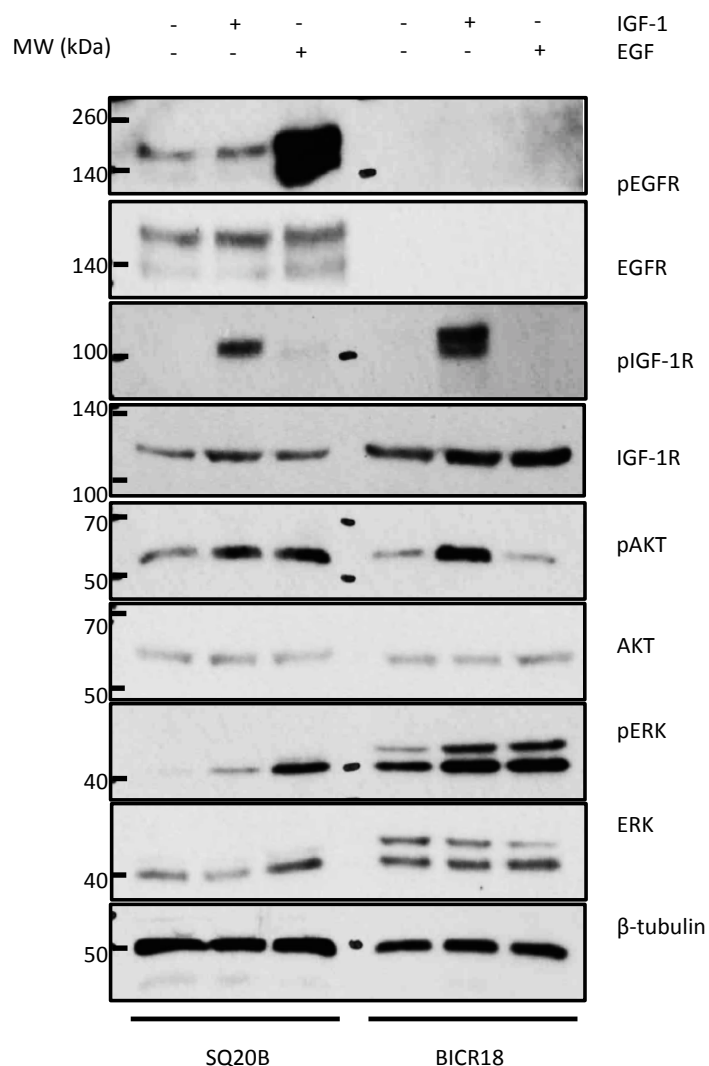
### 2.3.5 Statistical Analysis

All statistical analyses were pre-determined and performed using Prism 7 for Mac OS X (© 1994-2015 GraphPad Software, Inc). Radio sensitivity was determined by calculating the dose of irradiation required to suppress survival to 50% of irradiated controls (SF<sub>50</sub>) with 95% confidence intervals. The dose enhancement ratio (DER) at 2Gy and 5 Gy (where specified) was calculated when comparing the effects of receptor inhibition. This was taken as the ratio of control cells surviving at 2Gy or 5Gy vs. treated cells. Results of clonogenic survival assays were assessed using multiple t-tests (Holm-Sidak correction) or one-way ANOVA (Tukey's test) if >2 groups were compared. A result was considered significant when p was  $\leq 0.05$ .

## **2.4 Results:**

### 2.4.1 Establishing Baseline Characteristics of SQ20B and BICR18 cells

Western blots were performed to establish the baseline characteristics of SQ20B and BICR18 cells (**Figure 2-2**). The aim was to characterise the cell lines, confirm appropriate activation of targeted receptors, identify suitable targets for inhibition and determine the downstream effects of IGF-1R and EGFR stimulation.



**Figure 2-2:** Western blot comparing parental SQ20B and BICR18 cells

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1; EGFR, epidermal growth factor receptor.

*Equivalent amounts of protein were utilised to perform western blot to compare the relative expression of total and phosphorylated IGF-1R, EGFR, AKT, ERK and β-tubulin between parental SQ20B and parental BICR18 cells. Presented is a representative result from three independent assays with equivalent protein loading evidenced by equal β-tubulin expression. SQ20B and BICR18 cells were treated with IGF-1 (50nM) and EGF ligand (20nM) for 20 minutes before cells were lysed.*

Total and phosphorylated IGF-1R, EGFR, AKT and ERK were compared with and without the presence of IGF-1 and EGF ligand. SQ20B cells expressed IGF-1R and EGFR, whilst BICR18 expressed only IGF-1R.

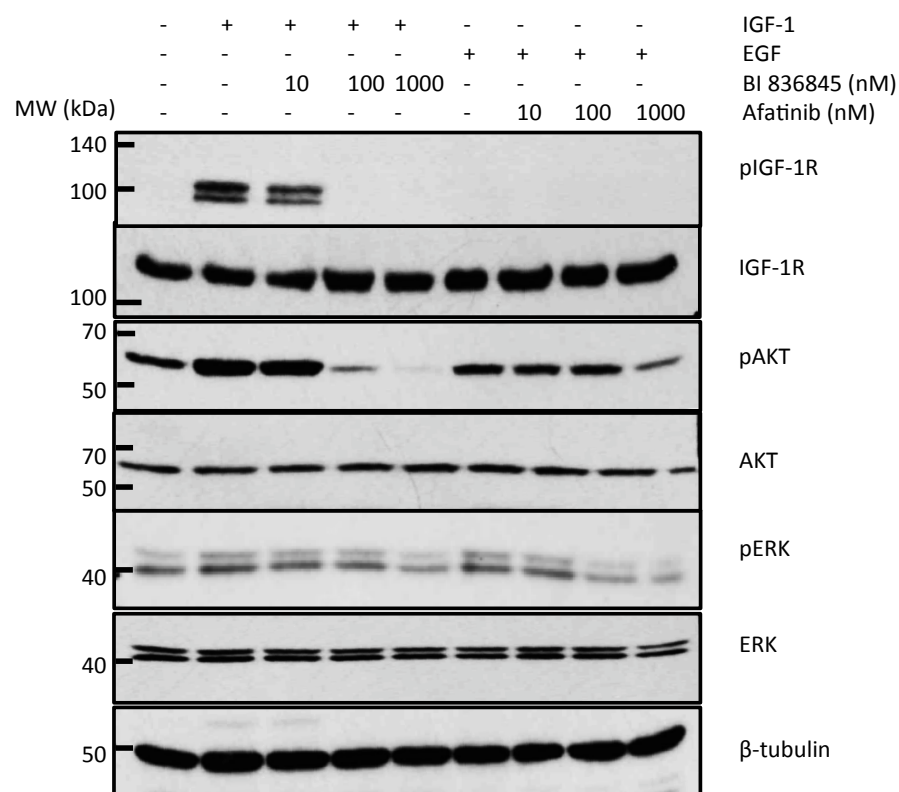
In SQ20B cells, IGF-1 resulted in activation of IGF-1R and downstream effectors AKT and ERK. EGF ligand resulted in phosphorylation of EGFR and downstream effectors AKT and ERK. There was also weak activation of IGF-1R; this relationship was later explored in detail (**see section 2.4.5**). In this cell line, EGF ligand had a greater effect on ERK phosphorylation than IGF-1.

In BICR18 cells, IGF-1 resulted in activation of IGF-1R and downstream effectors AKT and ERK. The effects of EGF-ligand on EGFR phosphorylation could not be assessed, as this receptor was not detected. However, EGF ligand stimulation resulted in activation of ERK. The signalling pathway for this effect remained undetected, but may have related to an alternative ErbB family receptor e.g. Her2. Subsequent western blotting for this receptor was unsuccessful.

The results confirmed the presence of EGFR and IGF-1R signalling cascades in SQ20B cells and IGF-1R signalling in BICR18 cells. It also meant there was biological evidence to support the existence of previously described IGF-1R and EGFR pathways (**Figure 2-1a**).

#### 2.4.2 Testing Effects of Combined IGF-1R and EGFR Inhibition in Laryngeal Cancer Cells.

Next, western blotting was performed to determine the effects of IGF-1R and EGFR inhibition on BICR18 and SQ20B cells. Both cell lines were treated with mono therapy (BI836845 or Afatinib) (**Figure 2-3 and 2-4**) and combination therapy (BI836845/Afatinib) (**Figure 2-5**). Although EGFR had not been detected in BICR18 cells, the activation of ERK in response to EGF-ligand suggested the presence of another ErbB family receptor. Given that Afatinib is known to affect this receptor family[113], the decision was made to investigate the effects of this drug on BICR18 cells also. The aim was to confirm target and downstream inhibition with both drugs, and determine a suitable concentration for subsequent clonogenic survival assays.

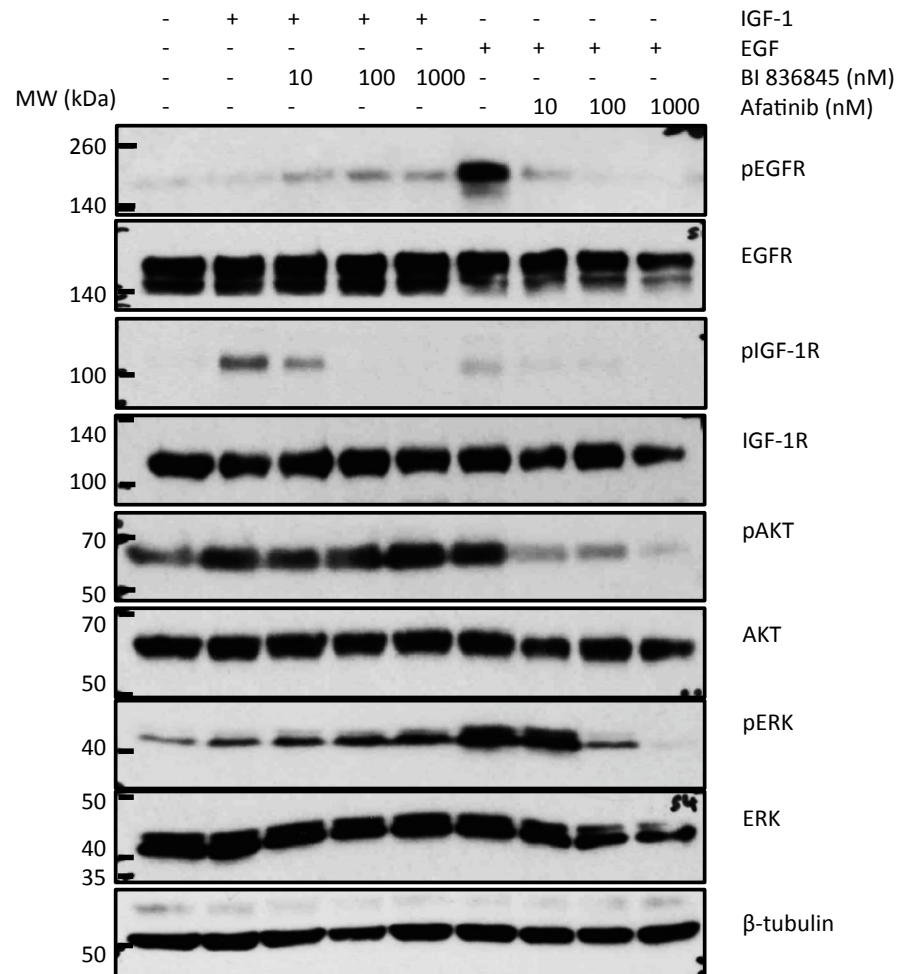


**Figure 2-3:** Western Blot comparing effects of IGF-1R and EGFR inhibition in BICR18 cells

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1.

*Western blotting to compare the relative expression of total and phosphorylated IGF-1R, EGFR, AKT, ERK and  $\beta$ -tubulin between BICR18 cells treated with BI836845, Afatinib or equivalent volume of solvent (dimethyl sulfoxide). Cells were lysed, and equal concentrations of protein used for western blotting as previously described (see methods). Presented is a representative result from three independent assays with equivalent protein loading evidenced by equal  $\beta$ -tubulin expression. These results demonstrate direct and downstream target inhibition of IGF-1R with BI836845 at 100nM and 1000nM. There is also dose dependant inhibition of AKT phosphorylation with increasing doses of BI836845 (100nM and 1000nM). Afatinib has no effect on IGF-1R, however it appears to cause dose dependant inhibition of ERK phosphorylation at 100nM and 1000nM.*

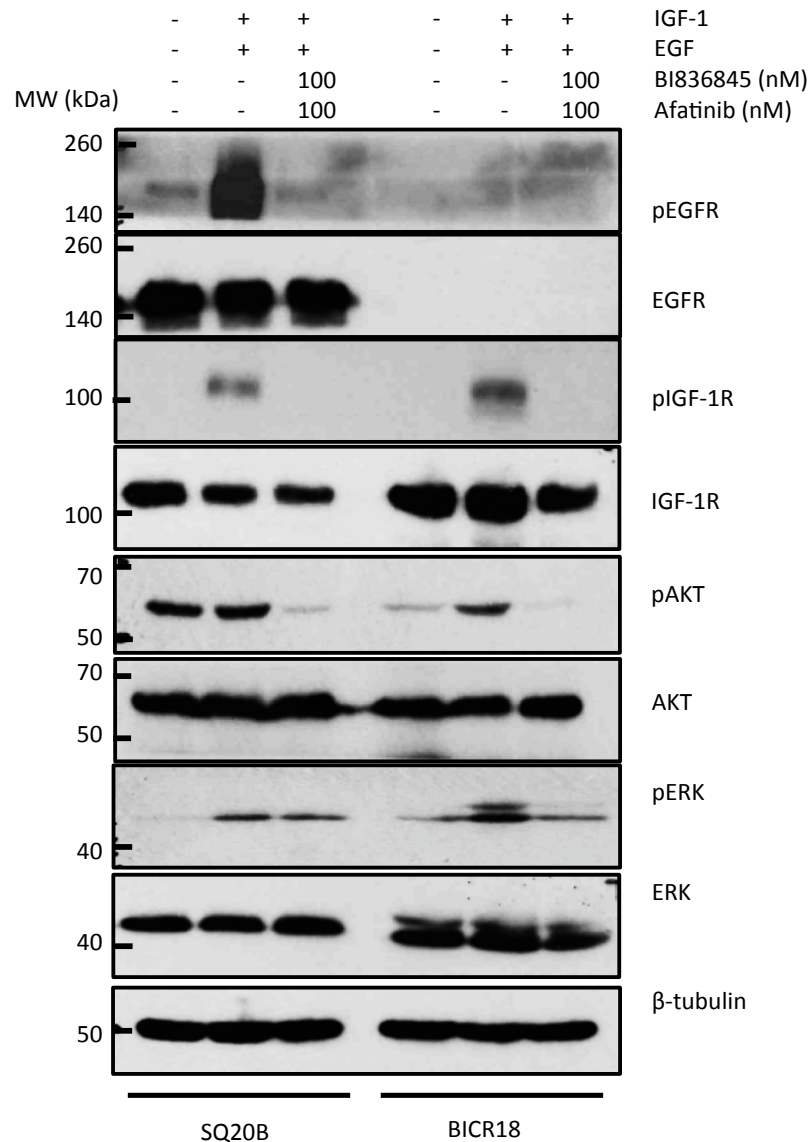




**Figure 2-4:** Western Blot comparing effects of IGF-1R and EGFR inhibition in SQ20B cells

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1; EGFR, epidermal growth factor receptor.

*Western blotting to compare the relative expression of total and phosphorylated IGF-1R, EGFR, AKT, ERK and β-tubulin between SQ20B cells treated as in figure 2-3. Presented is a representative result from three independent assays with equivalent protein loading evidenced by equal β-tubulin expression. BI836845 (10nM, 100nM and 1000nM) inhibits pIGF-1R but not its downstream targets (pERK or pAKT). Afatinib (10nM, 100nM, 1000nM) decreases pEGFR and pAKT but not pERK except at 100nM and 1000nM.*



**Figure 2-5:** Western Blot comparing effects of IGF-1R and EGFR combined inhibition on BICR18 and SQ20B cells

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1; EGFR, epidermal growth factor receptor.

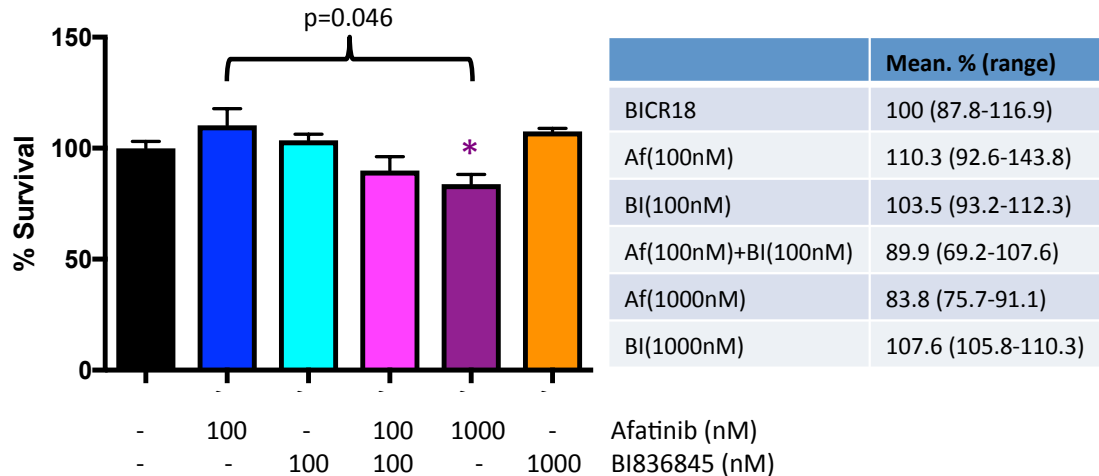
*Western blot to compare the relative expression of total and phosphorylated IGF-1R, EGFR, AKT, ERK and  $\beta$ -tubulin between SQ20B and BICR18 cells treated with both BI836845 and Afatinib at 100nM in the presence of both IGF and EGF ligand (as in legend for figure 2-3). Presented is a representative result from three independent assays with equivalent protein loads evidenced by equal  $\beta$ -tubulin expression. In SQ20B cells, combined treatment was effective at inhibiting pIGF-1R, pEGFR, pAKT but not pERK. Whilst in BICR18 cells combination treatment resulted in decreased pIGF-1R, pAKT and pERK.*

In BICR18 cells (**Figure 2-3 and 2-5**), BI836845 (10nM) did not affect IGF-1R phosphorylation. BI836845 (100nM) resulted in decreased phosphorylation of IGF-1R and AKT, whilst BI836845 (1000nM) also reduced ERK phosphorylation. Afatinib (10nM, 100nM, 1000nM) did not affect phosphorylation of IGF-1R or AKT but reduced ERK phosphorylation at 100nM and 1000nM. Combination treatment at 100nM resulted in decreased IGF-1R, AKT and ERK phosphorylation.

In SQ20B cells (**Figure 2-4 and 2-5**), which expressed both IGF-1R and EGFR, BI836845 reduced IGF-1R phosphorylation but did not affect AKT or ERK activation. Afatinib decreased EGFR and AKT phosphorylation from 10nM, whilst ERK activation was only reduced at 100nM and 1000nM. EGF ligand was noted to result in activation of IGF-1R suggesting the presence of receptor 'cross-talk'. Combination treatment was effective at inhibiting IGF-1R, EGFR, AKT phosphorylation but not ERK.

Thus, in both cell lines BI836845 and Afatinib (100nM and 1000nM) demonstrated an effect on IGF-1R and EGFR signalling. These concentrations were selected for subsequent CSAs aimed at determining the effects of each drug alone and in combination on cell survival and as a radio-sensitizing agent.

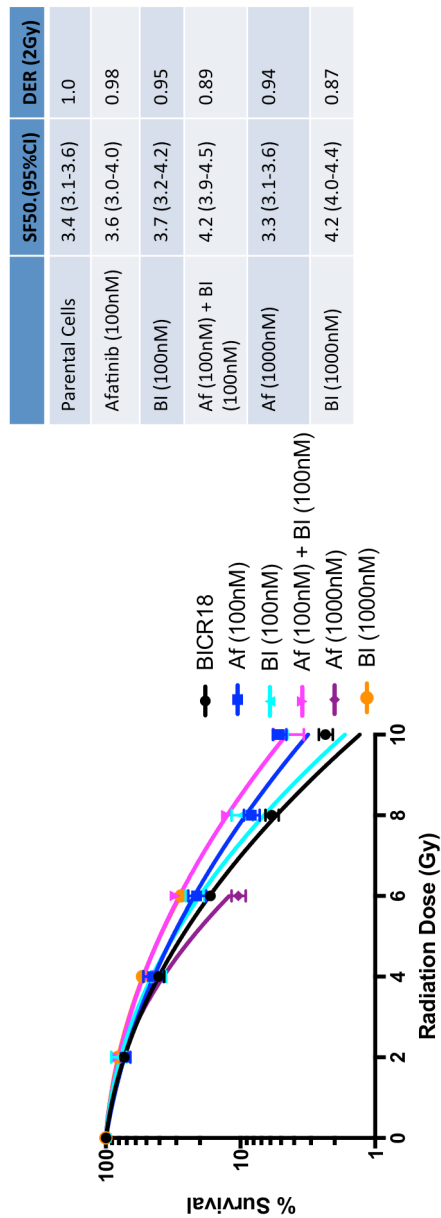
CSAs were performed to compare the effects of single and combined drug treatment in BICR18 and SQ20B parental cells (**Figure 2-6 and 2-7**).



**Figure 2-6a:** Clonogenic survival assay to assess effects of Afatinib and BI836846 alone and in combination on BICR18 cells surviving at 0Gy after 9 days (BICR18 cells vs. BICR18 cells treated with Afatinib and/or BI836845).

Abbreviations: Af, Afatinib; BI, BI836845

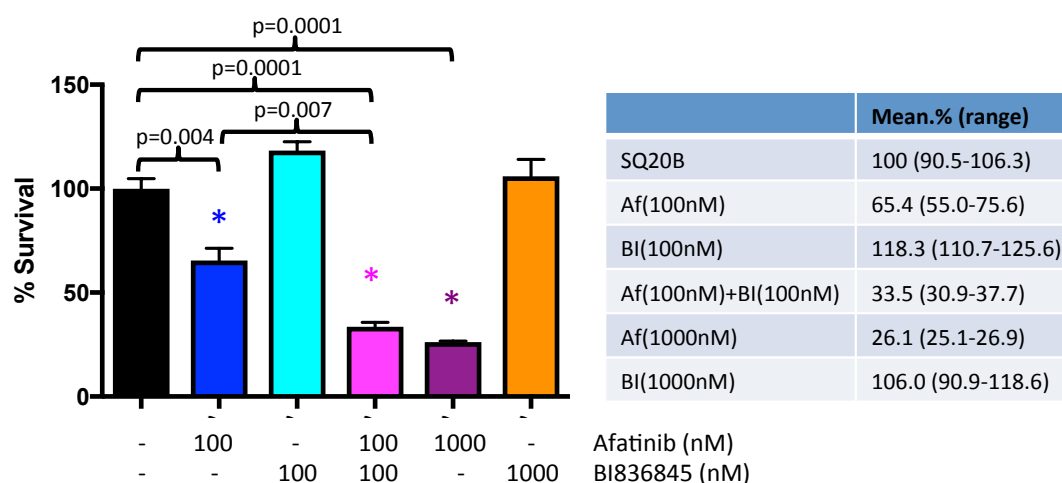
*For each drug combination tested, 2000 BICR18 cells were seeded into 100mm cell culture dishes and either drug solvent or drug treatment applied. 24 hours later, cells were treated with varying doses of radiotherapy (0-10Gy). The culture medium was replaced 24 hours later and cells allowed a further 7 days to grow before colony counting. This graph is a representative CSA from 2 independent assays performed in triplicate (except at 1000nM, which was only performed once) to compare colony survival in BICR18 cells treated with increasing doses of BI836845 and Afatinib as mono and combined therapy in the absence of radiation. There were significantly fewer colonies surviving at 9 days in cells treated with Afatinib 1000nM when compared to parental cells treated with Afatinib 100nM. Bars represent the number of colonies surviving as a proportion of parental cells  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between cell lines calculated with one-way ANOVA.*



**Figure 2-6b:** Clonogenic survival assay comparing BICR18 cells vs. BICR18 cells treated with Afatinib and/or BI836845 with increasing doses of radiotherapy.

Abbreviations: Af, Afatinib; BI, BI836845; DER, dose enhancement ratio; Gy, Gray; SF<sub>50</sub>, standard dose fraction for 50% cell survival

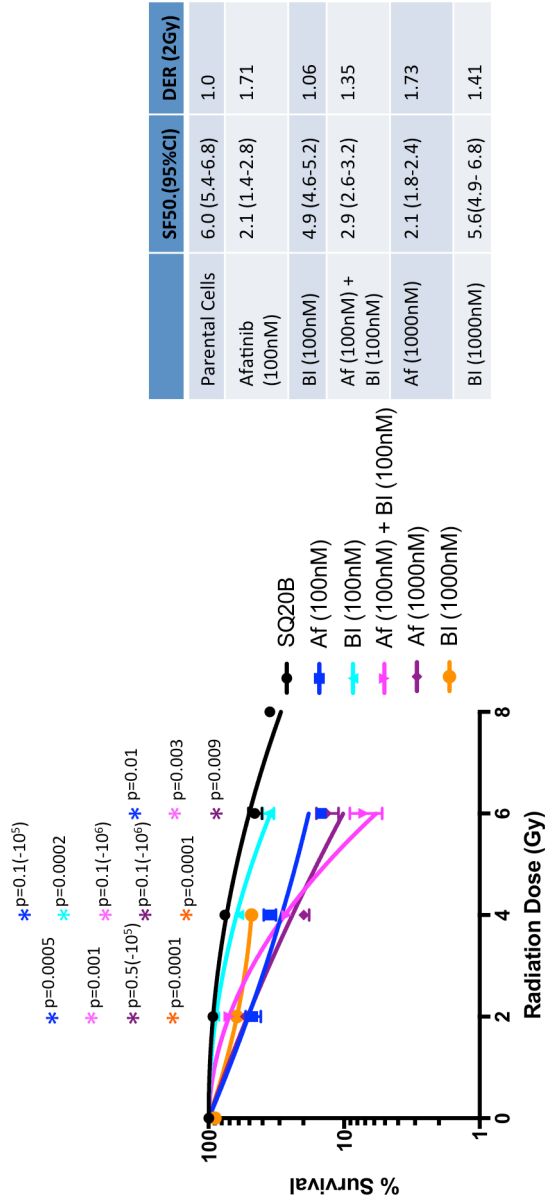
*This graph is a representative CSA from 2 independent assays performed in triplicate (except at 1000nM, which was only performed once) to compare colony survival in BICR18, treated with Afatinib, BI836845 or both alongside increasing doses of radiation (treatments were performed as described in Figure 2.6a). There was no difference in colony counts, SF<sub>50</sub> or DER at 2Gy when comparing cells treated with different drug regimes. Points represent the number of surviving colonies as a proportion of non-irradiated cells of the same type  $\pm$  standard error of the mean (SEM).*



**Figure 2-7a:** Clonogenic survival assay to show percentage of colonies surviving at 0Gy after 9 days (SQ20B cells vs. SQ20B cells treated with Afatinib and/or BI836845).

Abbreviations: Af, Afatinib; BI, BI836845

*This graph is a CSA from an assay performed with triplicate technical replicates, to compare baseline colony survival in SQ20B cells treated with increasing doses of BI836845 and Afatinib as mono and combined therapy in the absence of radiation (treatments were performed as described in Figure 2-6a). There were significantly fewer colonies surviving at 7 days in cells treated with Afatinib 100nM, Afatinib 1000nM and Afatinib/BI836845 100nM when compared to control-treated cells. Combined treatment with Afatinib and BI836845 had a greater effect on survival than Afatinib alone. Bars represent the number of colonies surviving as a proportion of parental cells  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between cell lines calculated with one-way ANOVA.*



**Figure 2-7b:** Clonogenic survival assay comparing SQ20B cells vs. SQ20B cells treated with Afatinib and/or BI836845 with increasing doses of radiotherapy.

Abbreviations: Af, Afatinib; BI, BI836845; DER, dose enhancement ratio; Gy, Gray; SF<sub>50</sub>, standard dose fraction for 50% cell survival

CSA from an assay performed with triplicate technical replicates, to compare colony survival in SQ20B cells treated with Afatinib, BI836845 or both alongside increasing doses of radiotherapy (as described in figure 2.6a). Both drugs appeared to enhance the effects of radiotherapy. Afatinib 100nM was superior to BI836845 100nM. Combination of BI836845 and Afatinib had no benefit over Afatinib alone. Points represent the number of surviving colonies as a proportion of untreated cells of the same type  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between cell lines calculated using multiple t-tests.

There was no difference in cell survival in BICR18 cells treated with 10-1000nM BI836845 or 100nM Afatinib, while Afatinib 1000nM reduced the mean colony count by 16% ( $p=0.046$ ) compared with solvent treated cells (**Figure 2-6a**). When BICR18 cells were treated with single and combined drug therapy alongside increasing doses of radiation there was no difference in terms of  $SF_{50}$ , DER at 2Gy, or survival at each radiotherapy dose. The results suggested that BICR18 cells were not radiosensitized by BI836845 or Afatinib alone or in combination (**Figure 2-6b**).

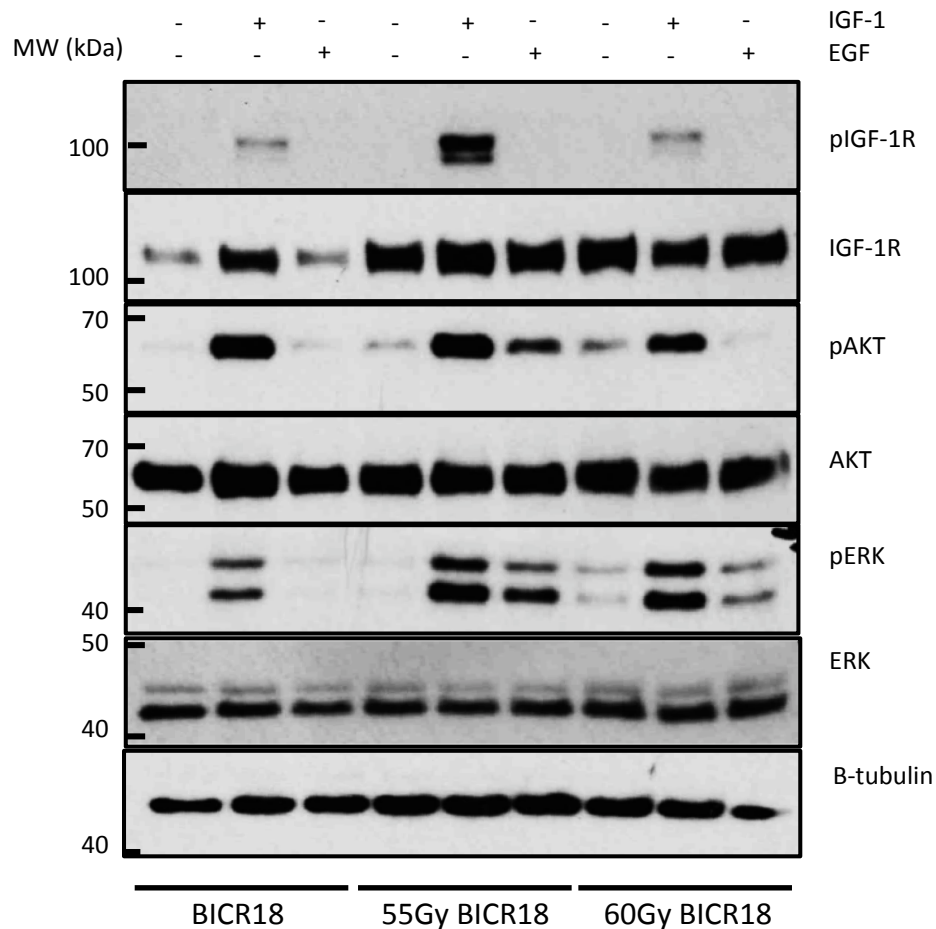
In SQ20B cells treatment with Afatinib 100nM, Afatinib 1000nM and BI836845/Afatinib 100nM was associated with a 35% ( $p=0.004$ ), 74% ( $p=0.0001$ ) and 67% ( $p=0.0001$ ) reduction in baseline colony survival at 0Gy, whilst BI836845 alone had no effect. There were 32% ( $p=0.007$ ) fewer colonies in cells treated with combined BI836845 and Afatinib at 100nM when compared to Afatinib 100nM alone (**Figure 2-7a**). However, this combined effect was inferior to the reduction in cell survival achieved by Afatinib at 1000nM.

In SQ20B cells treated with Afatinib alone and in combination with BI836845 there was evidence of radiosensitization, with reduction in  $SF_{50}$  and increase in DER at 2Gy. Treatment with Afatinib (100nM) was more effective than BI836845 at 1000nM but not at 100nM, and Afatinib (100nM) alone was as effective as Afatinib and BI836845 (100nM) in combination (**Figure 2-7b**).



#### 2.4.3 Effects of Repeated Irradiation on Laryngeal Cancer Cells.

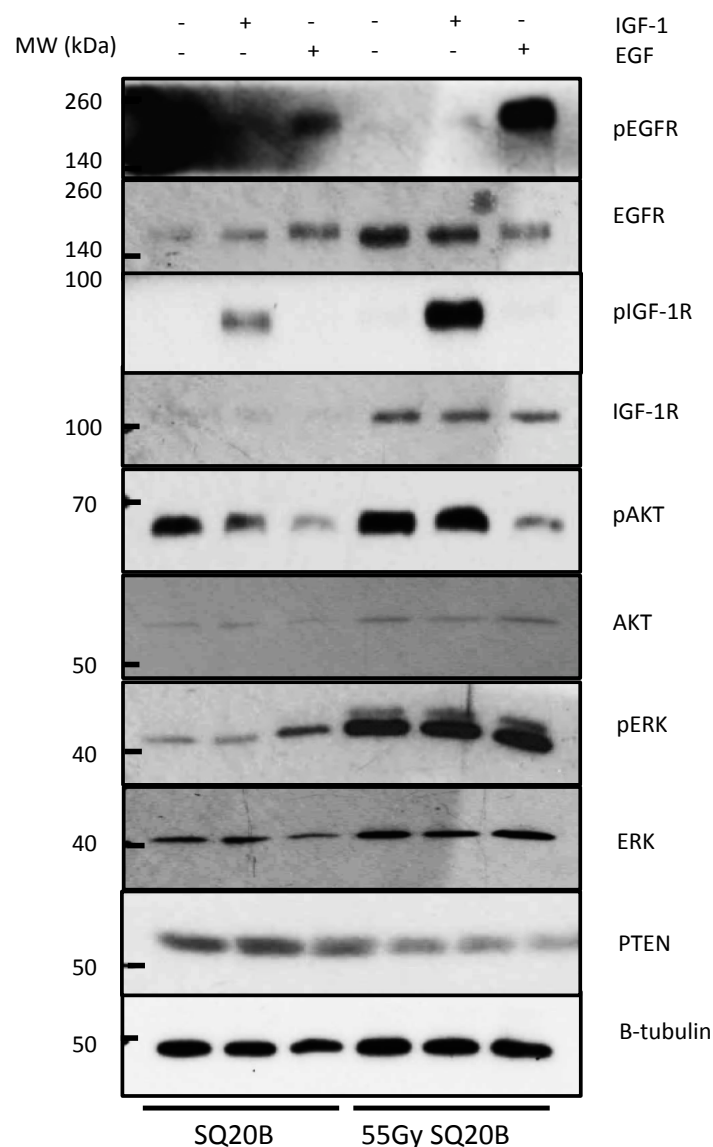
Both cell lines were repeatedly irradiated to assess changes induced by clinically used doses of radiation (see methods). Western blots were performed on parental and repeatedly irradiated cells to determine total and phosphorylated IGF-1R, EGFR and activation and expression of downstream targets (**Figure 2-8 and 2-9**).



**Figure 2-8:** Western Blot comparing BICR18, 55GyBICR18 and 60GyBICR18 cells

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1

*Western blotting to compare the relative expression of total and phosphorylated IGF-1R, EGFR, AKT, ERK and  $\beta$ -tubulin between parental BICR18 and repeatedly irradiated 55GyBICR18 and 60GyBICR18 cells. Presented is a representative result from three independent assays (performed as in Figure 2-2) with equivalent protein loading evidenced by equal  $\beta$ -tubulin expression. They demonstrate up regulation of baseline total IGF-1R and increased phosphorylation of AKT and ERK in response to IGF ligand in 55GyBICR18 and 60GyBICR18. They also suggest EGF ligand stimulation associates with phosphorylation of AKT in 55GyBICR18 and ERK in 55GyBICR18 and 60GyBICR18; this is not seen in the parental cell line.*



**Figure 2-9:** Western Blot to compare SQ20B and 55GySQ20B cells.

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1; EGFR, epidermal growth factor receptor.

*Western blotting to compare the relative expression of total and phosphorylated IGF-1R, EGFR, AKT, ERK, PTEN and  $\beta$ -tubulin between parental (SQ20B) and repeatedly irradiated cells (55GySQ20B). Presented is a representative result from three independent assays (performed as in Figure 2-2) with equivalent protein loading evidenced by equal  $\beta$ -tubulin expression. Exposure artefact is noted when interpreting phosphorylation of EGFR. These results demonstrate higher levels of EGFR, IGF-1R, ERK and lower PTEN in repeatedly irradiated cells (55GySQ20B). IGF-1 and EGF ligand stimulation has a greater effect on phosphorylation of IGF-1R and EGFR in irradiated cells when compared to parental cells.*

Parental BICR18 cells expressed IGF-1R, AKT, and ERK but not EGFR. IGF-1 stimulation was associated with phosphorylation of IGF-1R, AKT and ERK. EGF ligand stimulation had no effect on these targets. These results were similar to those seen earlier (Figure 2-2); however on this occasion EGF ligand was shown to activate ERK phosphorylation, the cause for this was unclear.

When compared, both irradiated BICR18 cell lines (55GyBICR18 and 60GyBICR18) expressed higher total IGF-1R than parental cells. IGF-1 had a greater effect on phosphorylation of IGF-1R in 55GyBICR18. IGF-1 also had a similar effect on phosphorylation of AKT and ERK in 55GyBICR18 and 60GyBICR18. EGF ligand resulted in phosphorylation of AKT in 55GyBICR18 and ERK in both 55GyBICR18 and 60GyBICR18, these effects were not seen in the parental cell line (**Figure 2-8**).

SQ20B parental cells expressed EGFR, IGF-1R, AKT, and ERK as previously seen (in **Figure 2-2**). IGF ligand stimulation resulted in phosphorylation of IGF-1R, whilst EGF ligand was associated with phosphorylation of EGFR and ERK. These results were again similar to those previously seen (**Figure 2-2**). Repeatedly irradiated cells (55GySQ20B) expressed higher levels of EGFR and IGF-1R when compared to parental cells (Western blots of 60GySQ20B failed due to technical issues and time constraints meant repeats were not possible). IGF-1 and EGF ligand had a greater effect on phosphorylation of IGF-1R and EGFR in irradiated cells when compared to parental cells. The irradiated cells also showed higher basal and IGF-1 induced AKT phosphorylation, and higher phosphorylated ERK than parental cells. In an attempt to identify reasons for elevated AKT phosphorylation, PTEN expression was assessed. This appeared to be lower in irradiated cells (**Figure 2-9**).

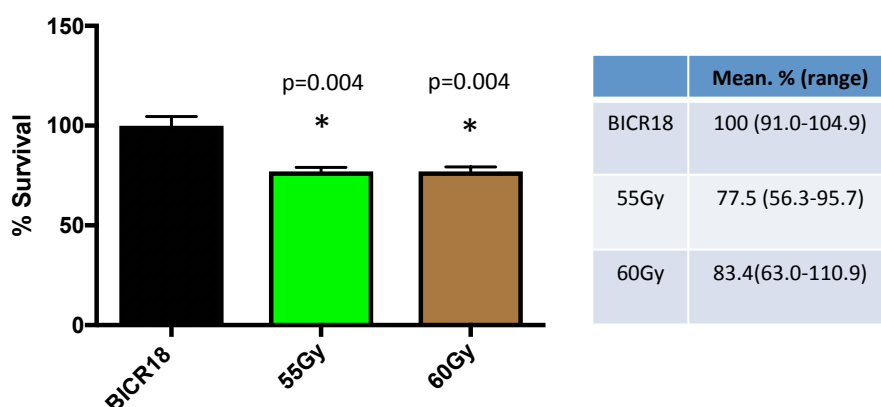
Next, clonogenic survival assays were performed to determine baseline survival for each parental and irradiated cell line (**Figure 2-10a and 2-11a**). Baseline colony counts in the absence of radiation were significantly lower for irradiated cells irrespective of irradiation regime or cell line ( $p < 0.05$ ). Specifically, baseline colony counts were 23% lower ( $p = 0.004$ ) for 55GyBICR18 and 60GyBICR18 when compared to BICR18. In irradiated SQ20B cells, baseline survival was low ( $< 30\%$ ); to overcome this 10,000 cells of each irradiated cell line were seeded and the assay repeated. The subsequent reduction in colony counts were less obvious but still significant. They were 10% ( $p = 0.015$ ) and 19% ( $p = 0.0004$ ) lower in 55GySQ20B and 60GySQ20B when compared to SQ20B. Pooled analysis, confirmed reduced baseline survival for irradiated SQ20B cells.

Next, the relative radio sensitivity of each parental and irradiated cell line was assessed (**Figure 2-10b and 2-11b**). The  $SF_{50}$  for 55GyBICR18 was higher than that of 60GyBICR18 and BICR18 (4.4 vs. 3.7 vs. 4.0) but the DERs at 2Gy were similar. At 8Gy both 60GyBICR18 and 55GyBICR18 had significantly higher survival rates ( $p = 0.007$  and  $p = 0.002$ ) whilst 60GyBICR18 also had improved survival at 10Gy ( $p = 0.001$ ). The results suggested a trend towards radiotherapy resistance in repeatedly irradiated laryngeal cancer cells 55GyBICR18 and 60GyBICR18.

Radiation survival assays in SQ20B cells indicated radioresistance in 55GySQ20B cells, with a higher  $SF_{50}$  and lower DER at 2 Gy than 60GySQ20B and SQ20B cells (4.3 and 0.89 vs. 2.5 and 1.2 vs. 3.3 and 1.0) (**Figure 2-11b**). Compared with parental cells, 60GySQ20B cells were more resistant at 2Gy ( $p = 0.005$ ), whilst 55GySQ20B cells were more resistant at 2, 4, 6, and 8Gy ( $p \leq 0.002$ ). Thus, there was evidence to suggest a

radiotherapy resistant phenotype in the repeatedly irradiated laryngeal cancer cell line 55GySQ20B.

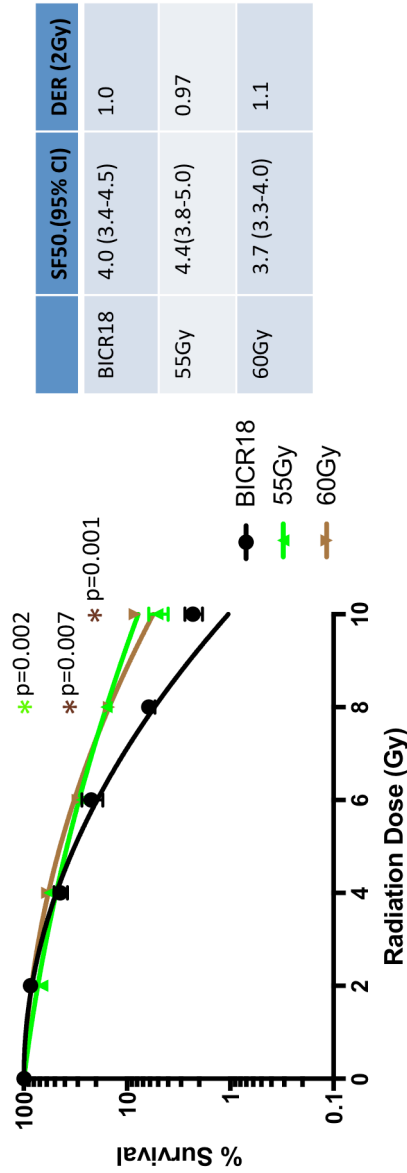
To test whether apparent radioresistance or changes in expression were due to a later passage of the irradiated cell line, CSAs were performed to compare early and late passage BICR18 and SQ20B cells (**Figure 2-10c and 2-11c**). The results demonstrated no statistically significant difference in relative radiosensitivity when comparing early and late passage BICR18 and SQ20B cells.



**Figure 2-10a:** Clonogenic survival assay to show percentage of colonies surviving at 0Gy after 7 days (BICR18 vs. 55GyBICR18 vs. 60GyBICR18).

Abbreviations: Gy, Gray; %, percentage.

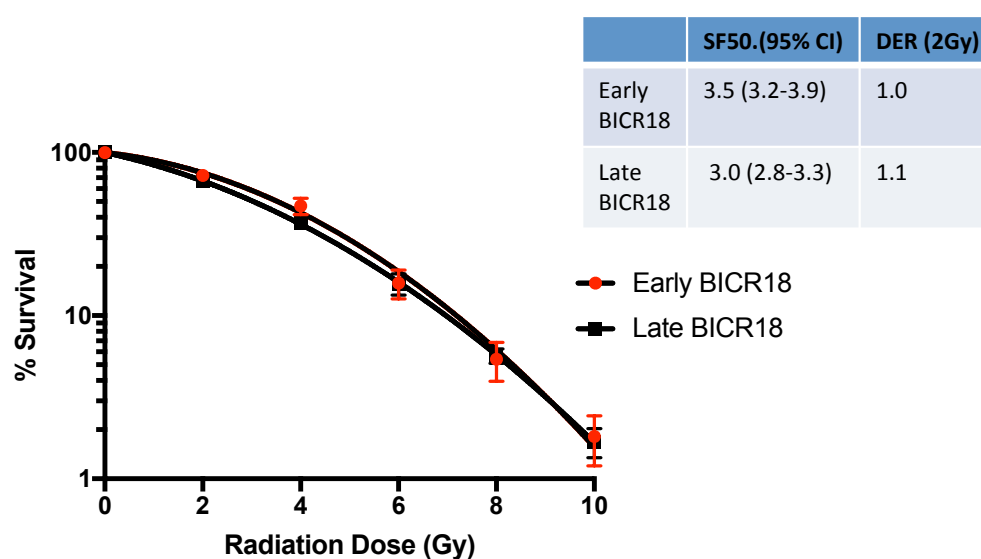
*For each cell line tested, 2000 cells were seeded into 100mm cell culture dishes. 24 hours later, cells were treated with varying doses of radiotherapy (0-10Gy). Cells were allowed a further 7 days before colony counting. Presented here is a representative CSA from one of 4 independent assays performed in triplicate to compare baseline colony survival in BICR18, 55GyBICR18 and 60GyBICR18 cells with no treatment at 7 days. The table presents the mean of all 4 assays. There were significantly fewer colonies surviving at 7 days in cells previously treated with both radiotherapy regimes (55Gy20Fr and 60Gy30Fr) when compared to parental cells. Bars represent number of colonies surviving as a proportion of parental cells  $\pm$  SEM. \* marks a statistically significant difference compared with parental cells (one way ANOVA).*



**Figure 2-10b:** Clonogenic survival assay comparing BICR18, 55GyBICR18 and 60GyBICR18 cells.

Abbreviations: Gy, Gray; %, percentage; CI, Confidence interval; SF<sub>50</sub>, standard dose fraction for 50% cell survival; DER, dose enhancement ratio at 2Gy.

Representative CSA from 4 independent assays performed in triplicate to compare colony survival in BICR18, 55GyBICR18 and 60GyBICR18 cells treated with increasing doses of radiotherapy. The graph demonstrates the percentage of colonies surviving for each cell line with increasing doses of radiotherapy (0-10Gy). The table presents the mean of all 3 assays. There is insufficient evidence to conclude that repeated irradiation in BICR18 cells resulted in a radiotherapy resistant phenotype. DERs at 2Gy and SF<sub>50</sub>'s were similar for each cell line. Points represent the number of surviving colonies as a proportion of non-irradiated cells of the same type  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between the irradiated siblings and parental cells (multiple t-tests).

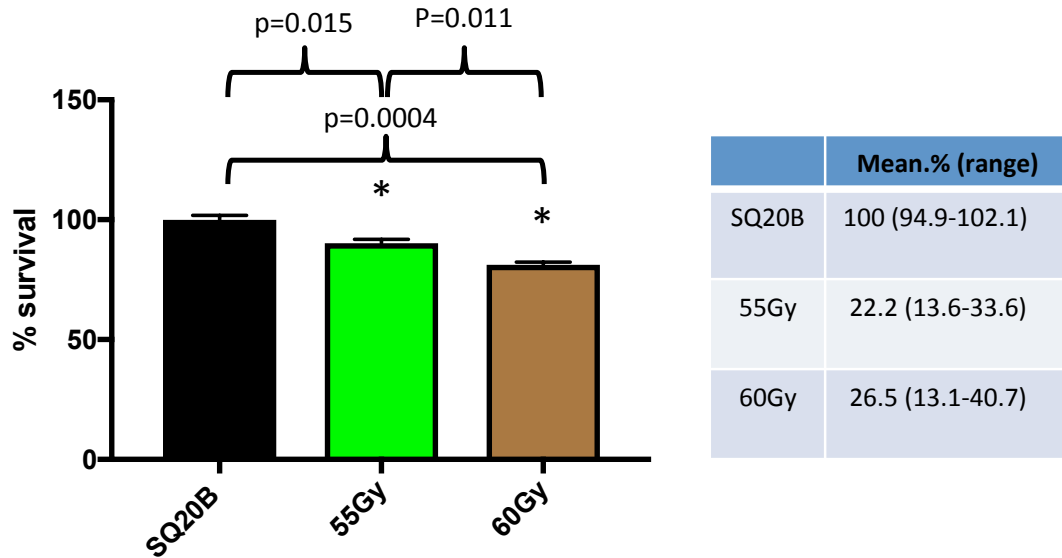


**Figure 2-10c:** Clonogenic survival assay comparing early vs. late passage BICR18 cells

Abbreviations: Gy, Gray; %, percentage; CI, Confidence interval; SF<sub>50</sub>, standard dose fraction for 50% cell survival; DER, dose enhancement ratio at 2Gy.

*CSA from 3 independent assays performed in triplicate to compare colony survival in early vs. late passage BICR18 cells (10 passage difference between early and late cells). The table presents the mean of all 3 assays. The graph demonstrates the percentage of colonies surviving for each cell line with increasing doses of irradiation (0-10Gy). There was no statistically significant difference (multiple t-tests) in overall and colony survival at each radiation dose. Points represent the number of surviving colonies as a proportion of non-irradiated cells of the same type  $\pm$  standard error of the mean (SEM).*

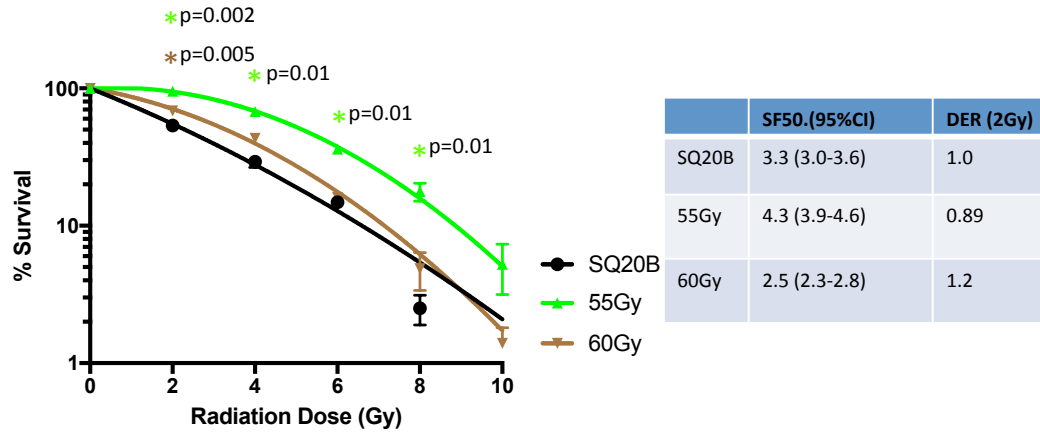




**Figure 2-11a:** Clonogenic survival assay to show percentage of colonies surviving at 0Gy after 7 days (SQ20B vs. 55GySQ20B vs. 60GySQ20B).

Abbreviations: Gy, Gray; %, percentage.

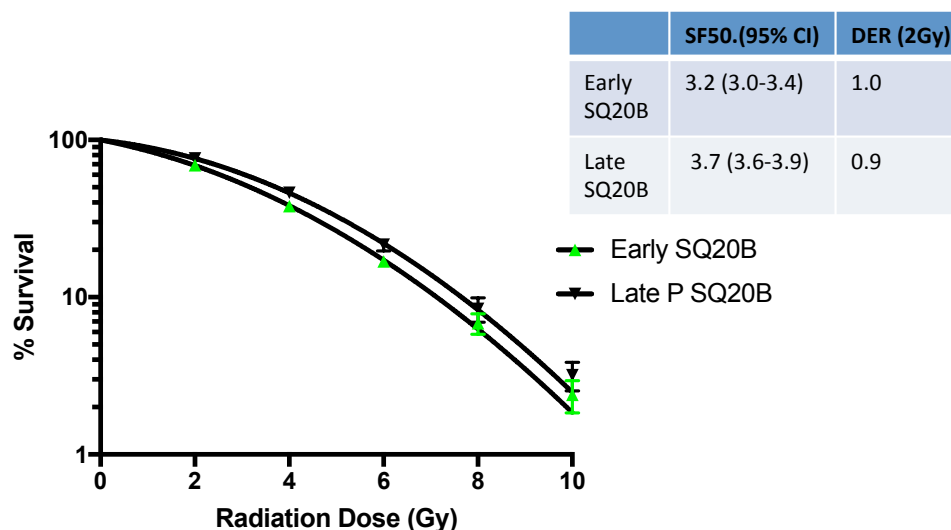
*Representative CSA from 4 independent assays (treated as in Figure 2-10a) performed in triplicate to compare baseline colony survival in non-irradiated SQ20B, 55GySQ20B and 60GySQ20B cells. In the presented assay, 10,000 cells of 55GySQ20B and 60GySQ20B cells were seeded, due to the comparatively poor survival at baseline noted for irradiated cells. The table presents the mean of the 3 assays performed when 2000 cells were seeded for each cell line. There were significantly fewer colonies surviving at 7 days in cells previously treated with both radiotherapy regimes (55Gy20Fr and 60Gy30Fr) when compared to parental cells. There were significantly fewer surviving colonies in cells treated with 60Gy30Fr when compared to those receiving 55Gy in 20Fr. This difference was not noted in pooled analysis. Bars represent the number of colonies surviving as a proportion of parental cells  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between cell lines calculated with one-way ANOVA.*



**Figure 2-11b:** Clonogenic survival assay comparing SQ20B, 55GySQ20B and 60GySQ20B cells.

Abbreviations: Gy, Gray; %, percentage; CI, Confidence interval; SF<sub>50</sub>, standard dose fraction for 50% cell survival; DER, dose enhancement ratio at 2Gy.

*Representative CSA from 4 independent assays performed in triplicate to compare colony survival in SQ20B, 55GySQ20B and 60GySQ20B cells treated with increasing doses of irradiation. The graph demonstrates the percentage of colonies surviving for each cell line with increasing doses of radiotherapy (0-10Gy). The table presents the mean of all 4 assays. Repeatedly irradiated 55GySQ20B cells had a higher SF50 and lower DER at 2Gy when compared to parental cells. 55GySQ20B appeared to demonstrate a radiotherapy resistant phenotype. Points represent the number of surviving colonies as a proportion of untreated cells of the same type  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between cell lines calculated using multiple t-tests.*



**Figure 2-11c:** Clonogenic survival assay comparing early vs. late passage SQ20B cells

Abbreviations: Gy, Gray; %, percentage; CI, Confidence interval; SF<sub>50</sub>, standard dose fraction for 50% cell survival; DER, dose enhancement ratio at 2Gy.

*Representative CSA from 3 independent assays performed in triplicate to compare colony survival in early vs. late passage SQ20B cells (10 passage difference between early and late cells). The table presents the mean of all 3 assays. The graph demonstrates the percentage of colonies surviving for each cell line with increasing doses of radiotherapy (0-10Gy). There was no statistically significant difference (multiple t-tests) in overall and colony survival at each radiation dose. Points represent the number of surviving colonies as a proportion of non-irradiated cells of the same type  $\pm$  standard error of the mean (SEM).*

#### 2.4.4 Testing Effects of Combined IGF-1R and EGFR Inhibition in Repeatedly

##### Irradiated Laryngeal Cancer Cells.

Following repeated irradiation, and its effect on IGF-1R and EGFR (SQ20B cells only) signalling, parental and irradiated cells were treated with BI836845 and Afatinib.

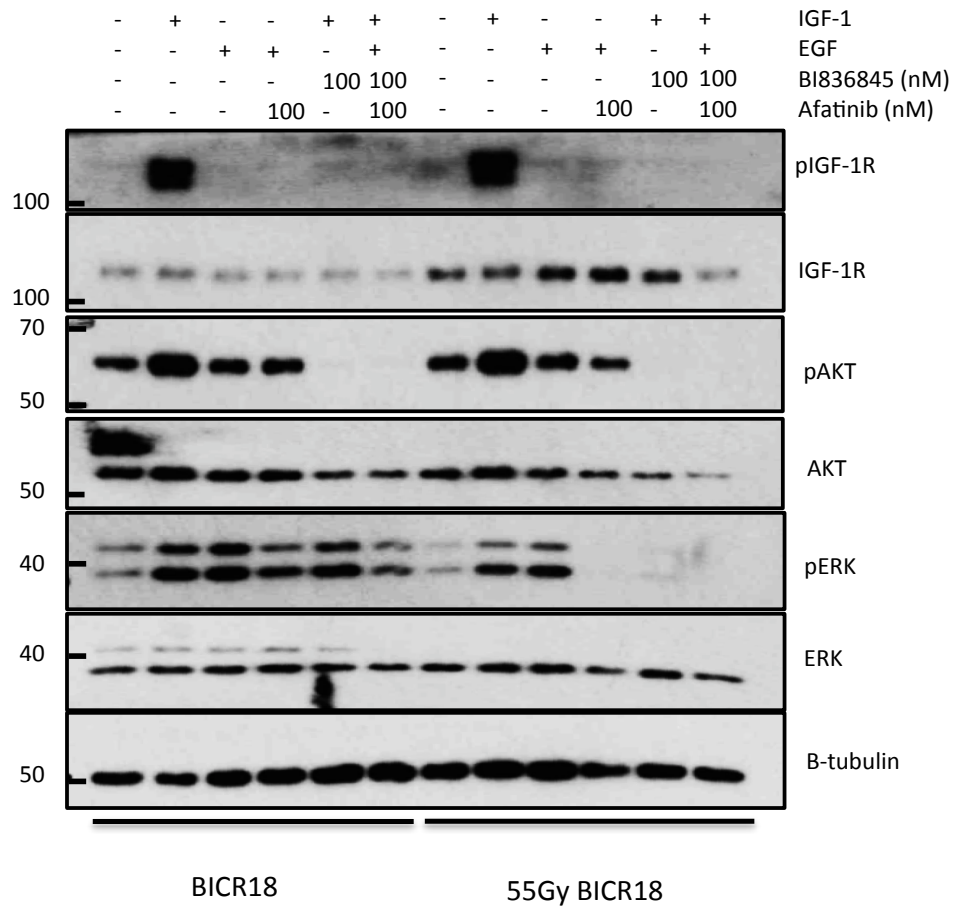
Western blots were conducted to assess the effects of Afatinib and BI836845 alone and in combination on BICR18 and 55GyBICR18 cells (**Figure 2-12a**). IGF-1R up regulation was again noted in the irradiated cells. Both drugs demonstrated target inhibition as previously seen (**Figure 2-3, 2-4, 2-5**). In 55GyBICR18 cells ERK

phosphorylation appeared to be more sensitive to inhibition, being inhibited by BI836845 alone and in combination with Afatinib. Afatinib alone did not have this effect. In contrast, the parental cells showed no ERK inhibition in response to BI836845, and minor reduction upon Afatinib treatment (**Figure 2-12a**), consistent with previously described results (**Figure 2-3**).

CSAs were performed to compare cell survival in response to single and combined drug treatment in the absence of irradiation (**Figure 2-12b**). Parental BICR18 cells showed no difference in survival when treated with BI836845 or Afatinib at 100nM (as in **Figure 2-6a**). In 55GyBICR18 cells each drug alone had no statistically significant effect on survival, however combined treatment resulted in a 28% lower colony count, which was significant when compared with untreated controls ( $p=0.032$ ). The relative radiosensitivity of BICR18 and 55GyBICR18 cells with single and combined treatment was also tested using CSA (**Figure 2-12c**). There was no detectable difference in response to irradiation when comparing cell lines or treatments at the doses tested.

SQ20B and 55GySQ20B cells were also compared for baseline survival when treated with both drugs in the absence of irradiation (**Figure 2-13a**). BI836845 alone did not inhibit cell survival in the parental or irradiated cells. Cell survival was significantly reduced in both SQ20B and 55GySQ20B cell lines when treated with Afatinib alone or in combination with BI836845. When relative survival in SQ20B cells and 55GySQ20B cells treated with both drugs was compared, it was apparent that 55GySQ20B cells were significantly more sensitive to combined IGF-1R/EGFR inhibition ( $p=0.0001$ ) (**Figure 2-13a**). This suggested a potential advantage to combination treatment in the repeatedly irradiated cells with IGF-1R up regulation.

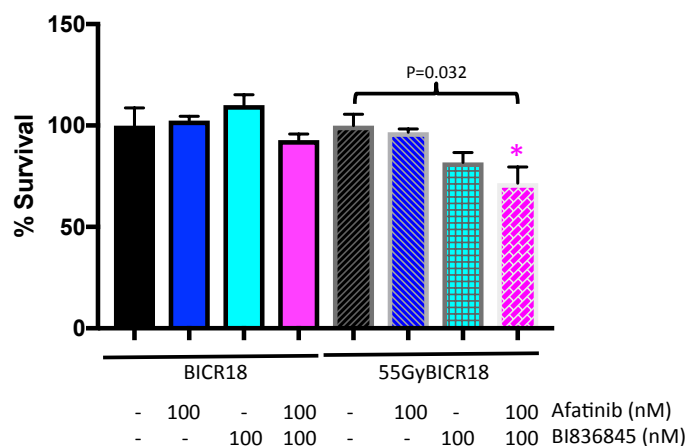
Radiation survival assays were then performed to determine whether mono or combination therapy was able to influence radiosensitivity of the parental and repeatedly irradiated SQ20B laryngeal cancer cell lines (**Figure 2-13b**). As before (in **Figure 2-7b**), BI836845 was ineffective, and parental cells were only modestly radiosensitized by Afatinib alone and with BI836845. The proportionate colony counts for SQ20B and 55GyS20B at 5Gy and 10Gy were not significantly different irrespective of treatment.



**Figure 2-12a:** Western blot to assess effects of BI836845 and Afatinib on BICR18 cells (BICR18 vs. 55GyBICR18).

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1; EGFR, epidermal growth factor receptor.

*Western blots were used to compare the relative expression of total and phosphorylated IGF-1R, AKT, ERK, and  $\beta$ -tubulin between parental (BICR18) and repeatedly irradiated cells (55GyBICR18) in response to IGF and EGFR inhibition (cells treated as per Figure 2.3). Presented is a representative result from two independent assays. Exposure artefact is noted when interpreting AKT. These results demonstrate higher levels IGF-1R and reduced pERK in response to BI836845 100nM and BI836845/Afatinib 100nM in repeatedly irradiated cells (55GyBICR18).*

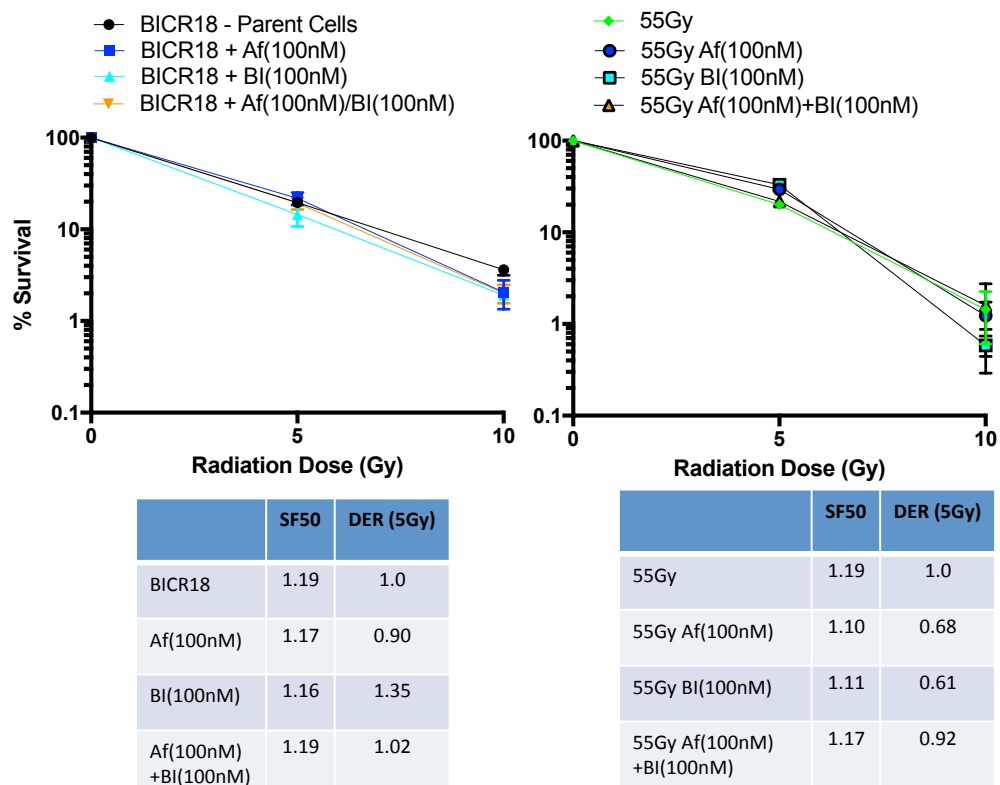


	Mean.% (range)
BICR18	100 (87.5-116.6)
Af(100nM)	102.5 (98.7-106.1)
BI(100nM)	110.1 (100.1-116.6)
Af(100nM)+BI(100nM)	92.9 (88.9-98.7)
55Gy	100 (90.9-110.3)
55Gy Af(100nM)	96.7 (93.8-99.5)
55Gy BI(100nM)	81.9 (74.5-90.9)
55Gy Af(100nM)+BI(100nM)	71.6 (56.8-84.1)

**Figure 2-12b:** Clonogenic survival assay to show percentage of colonies surviving at 0Gy after 9 days (BICR18 cells vs. 55GyBICR18 cells treated with Afatinib and/or BI836845).

Abbreviations: Af, Afatinib; BI, BI836845

CSA from a single assay performed in triplicate to compare baseline colony survival in BICR18 and 55GyBICR18 cells treated with BI836845 and Afatinib as mono and combined therapy in the absence of radiation (cells treated as per Figure 2.6a). There were significantly fewer colonies surviving at 9 days in 55GyBICR18 cells treated with Afatinib/BI836845 100nM when compared to 55GyBICR18 cells. There were no other significant differences in colony survival detected when comparing the remaining groups. These results suggested a potential benefit for combination treatment over mono therapy. Bars represent the number of colonies surviving as a proportion of parental cells  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between cell lines calculated with one-way ANOVA.

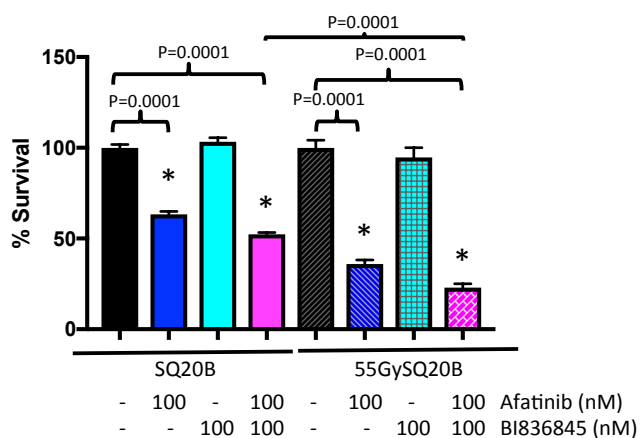


**Figure 2-12c:** Clonogenic survival assay comparing BICR18 cells vs. 55GyBICR18 cells treated with Afatinib and/or BI836845 with increasing doses of radiotherapy

Abbreviations: Af, Afatinib; BI, BI836845; DER, dose enhancement ratio; Gy, Gray; SF<sub>50</sub>, standard dose fraction for 50% cell survival

CSA from a single assay performed in triplicate to compare colony survival in BICR18 and 55GyBICR18 cells treated with Afatinib, BI836845 or both alongside increasing doses of radiotherapy (treatments given as in Figure 2-6a). Drug treatment did not enhance the effects of radiotherapy in parental cells. There was no difference in colony count, SF<sub>50</sub> or DER at 5Gy when comparing cell lines and treatment regimes. Points represent the number of surviving colonies as a proportion of untreated cells of the same type  $\pm$  standard error of the mean (SEM). Statistical comparisons were made using multiple t-tests.



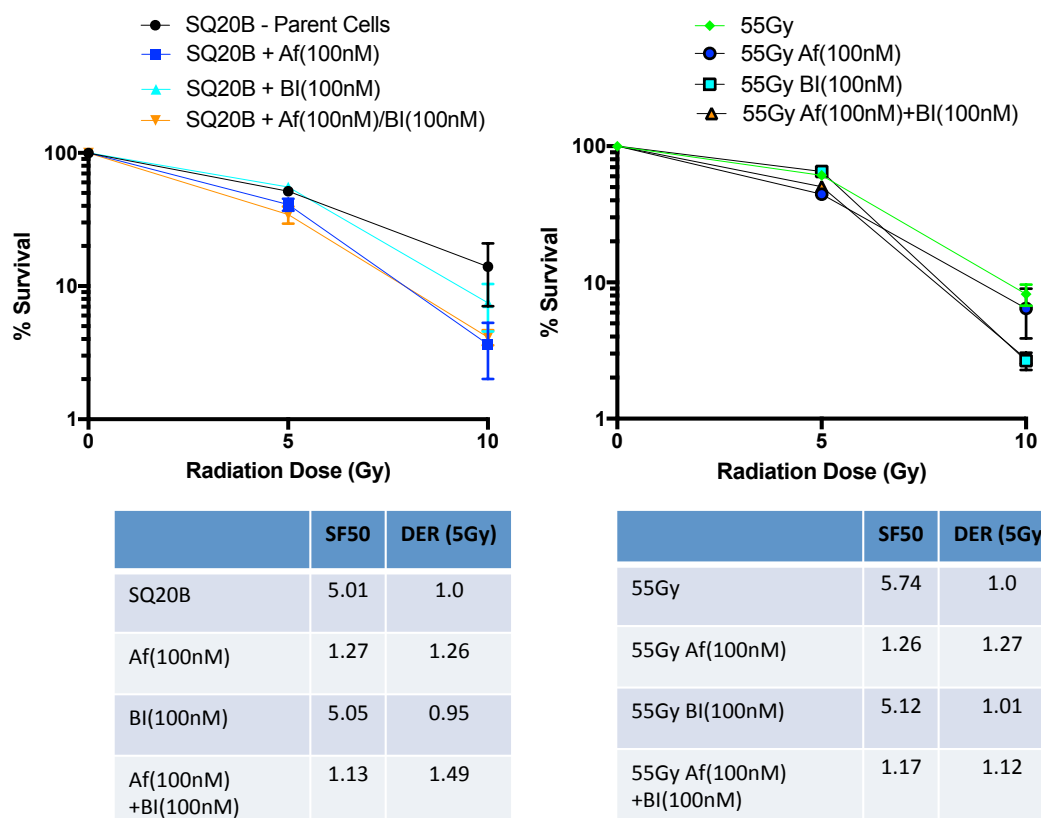


	Mean.% (range)
SQ20B	100 (97.8-103.6)
Af(100nM)	63.3 (60.9-66.5)
BI(100nM)	103.3 (99.1-106.6)
Af(100nM)+BI(100nM)	52.4 (50.5-53.5)
55Gy	100 (92.0-106.5)
55Gy Af(100nM)	35.9 (31.5-38.4)
55Gy BI(100nM)	94.7 (88.9-105.2)
55Gy Af(100nM)+BI(100nM)	22.9 (18.9-26.5)

**Figure 2-13a:** Clonogenic survival assay to show percentage of colonies surviving at 0Gy after 9 days (SQ20B cells vs. 55GySQ20B cells treated with Afatinib and/or BI836845).

Abbreviations: Af, Afatinib; BI, BI836845

CSA from a single assay performed (as in Figure 2-6a) in triplicate to compare baseline colony survival in SQ20B and 55GySQ20B cells treated with BI836845 and Afatinib as mono and combined therapy in the absence of radiation. There were significantly fewer colonies surviving at 9 days in parental cells treated with Afatinib 100nM and Afatinib/BI836845 100nM when compared to parental control cells. There was a similar result for 55GySQ20B cells. Although combination treatment appeared better in both cell lines when compared to Afatinib alone the difference was not statistically significant. There were significantly fewer colonies surviving for 55GySQ20B cells treated with combined therapy when compared to parental cells receiving the same treatment ( $p=0.0001$ ). These results suggested a potential benefit for combination treatment over mono therapy in cells with up regulation of IGF-1R. There were no other significant differences in colony survival detected when comparing the remaining groups. Bars represent the number of colonies surviving as a proportion of parental cells  $\pm$  standard error of the mean (SEM). \* marks a statistically significant difference between cell lines calculated with one-way ANOVA.



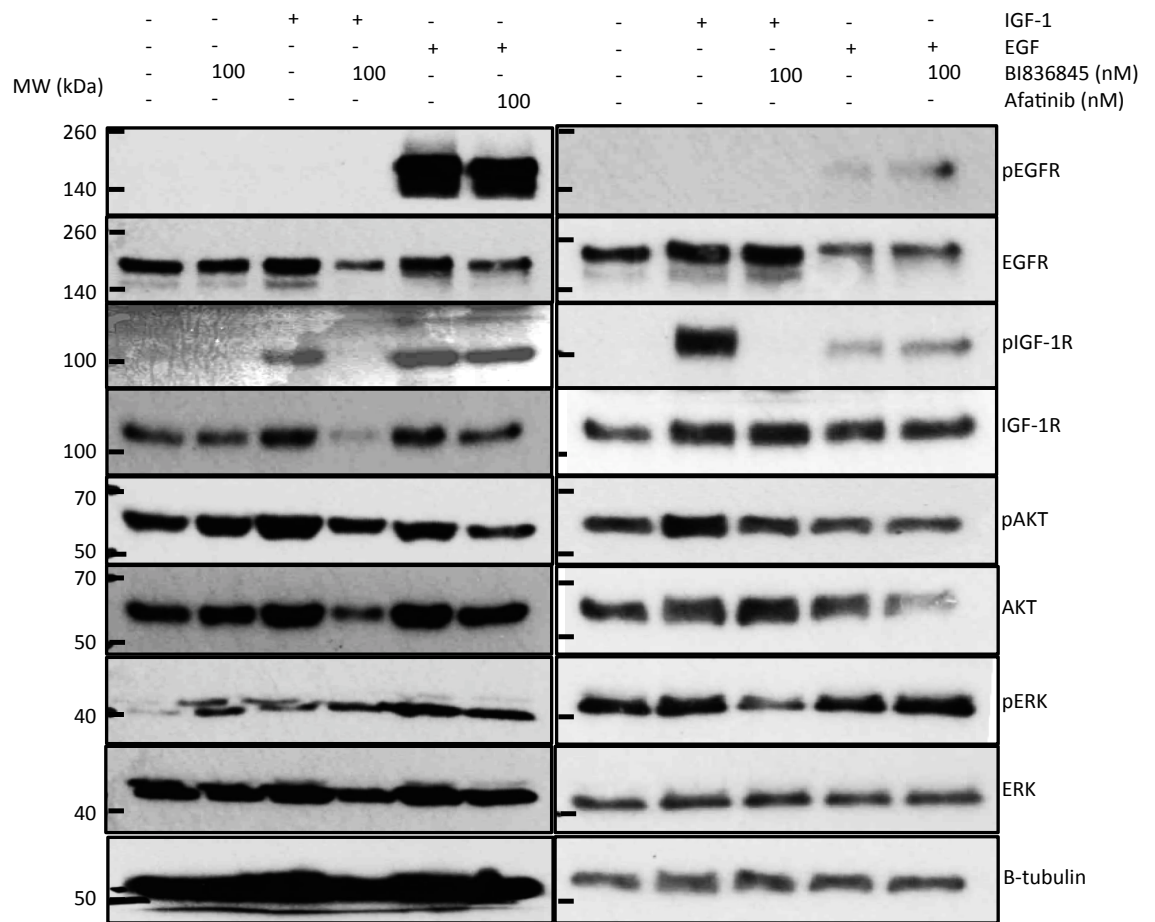
**Figure 2-13b:** Clonogenic survival assay comparing SQ20B cells vs. 55GySQ20B cells treated with Afatinib and/or BI836845 with increasing doses of radiotherapy

Abbreviations: Af, Afatinib; BI, BI836845; DER, dose enhancement ratio; Gy, Gray; SF<sub>50</sub>, standard dose fraction for 50% cell survival

CSA from a single assay performed in triplicate to compare colony survival in SQ20B and 55GySQ20B cells treated with Afatinib, BI836845 or both alongside increasing doses of radiotherapy (treatments given as in Figure 2-6a). The SF<sub>50</sub> was lower for both cell lines when treated with Afatinib alone and in combination with BI836845 in comparison to no treatment or treatment with BI836845 alone. The DERs at 2Gy and colony counts at each radiotherapy dose remained similar irrespective of treatment regime. Points represent the number of surviving colonies as a proportion of untreated cells of the same type  $\pm$  standard error of the mean (SEM). Statistical comparisons were made using multiple *t*-tests.

#### 2.4.5 Detecting Cross-Talk in SQ20B cells.

In SQ20B cells, EGF ligand was noted to result in increased IGF-1R phosphorylation (**Figure 2-4**). This potential relationship suggesting cross talk between the two receptors was explored using western blotting (**Figure 2-14**). Stimulation with EGF ligand resulted in phosphorylation of IGF-1R, but IGF-1 did not have this effect on EGFR. Afatinib did not block this phosphorylation, and when BI836845 was used alongside EGF ligand in an attempt to inhibit any IGF ligand mediated IGF-1R activation, there was no effect on IGF-1R, AKT or ERK phosphorylation. These results suggested an alternative mechanism for signalling to IGF-1R, for example through a co-receptor as previously described (**Figure 2-1b**).



**Figure 2-14:** Investigating cross-talk between EGFR and IGF-1R in SQ20B cells

Abbreviations: MW, molecular weight; kDa, kilodaltons; p, phosphorylated; EGF, epidermal growth factor; IGF-1, insulin like growth factor type 1, IGF-1R, insulin like growth factor receptor type 1; EGFR, epidermal growth factor receptor.

*These western blots compare the relative expression of total and phosphorylated IGF-1R, EGFR, AKT, ERK and  $\beta$ -tubulin in SQ20B cells in the presence of IGF-ligand, EGF ligand and Afatinib and BI836945. Presented is a representative result from three independent blots (performed as per Figure 2-3). Seen here is evidence of 'cross-talk' between the IGF-1R and EGFR. EGF ligand is seen to result in phosphorylation of IGF-1R. This effect is not inhibited by the use of Afatinib (EGF RTK) or BI836845. These results suggest EGF ligand in this cell line may directly result in stimulation of IGF-1R without the need for IGF-1. IGF ligand does not result in phosphorylation of EGFR.*

## 2.5 Discussion:

This study aimed to create an in vitro model of radiotherapy resistant laryngeal cancer cells using repeated irradiation. The overall aim was to compare expression and activation of EGFR, IGF-1R and communal downstream targets between parental and irradiated cells, and to determine whether irradiated or radiotherapy resistant cells responded differently to inhibition of the IGF and EGF axes.

Two different laryngeal cancer cell lines, BICR18 and SQ20B were used. Western blots showed that BICR18 cells expressed IGF-1R but not EGFR, whilst SQ20B cells expressed both. This was an unexpected result, as EGFR is frequently detected in HNSCC [100], as found in our earlier study (Chapter 1). LSCC cells were treated with two different irradiation regimens (55Gy20Fr and 60Gr30Fr) commonly used in clinical practise in an attempt to induce a radiotherapy resistant phenotype.

### 2.5.1 Developing a Model of Radiation Resistance in Laryngeal Cancer Cells

Repeatedly irradiated BICR18 cells showed a minor trend towards radiotherapy resistance (**Figure 2-10b**). For SQ20B cells repeated irradiation resulted in a radiotherapy resistant phenotype, with a higher SF<sub>50</sub> (4.3 vs. 3.3) and lower DER at 2Gy (0.89 vs. 1.0) in 55GySQ20B cells when compared to parental cells (**Figure 2-11b**).

Radiotherapy resistant cell lines have previously been developed to study a number of different solid organ tumours, including oesophageal [114], prostate [115], breast [116] and oral cancer [117]. A variety of protocols using repeated radiation exposure have been described, differing in overall total dose, length of treatment and recovery time [118]. Studies have utilised an average total dose of 40-60Gy [118] with treatment

duration ranging from 5 days [115] to 6 years [119] and 12-14 days recovery time after final treatment fraction. Lee et al (2013) [117] used a total of 60Gy to induce radiotherapy resistance in oral squamous cell carcinoma cell lines. As with our study, they based the dosing regime on current clinical practise. They concluded that the ideal study of radiation resistance in head and neck cancer should closely resemble a 'real clinical setting'. Given that most head and neck cancers are treated with 2-3Gy fractions up to a total of 50-70Gy, they selected a total dose of 60Gy. They did not specify the length of treatment and recovery time provided to cells. In the current study, radiotherapy regimes matched two protocols commonly used in our hospital for the treatment of LSCC. Cells were treated for 5 days followed by 2 days rest for 4 to 6 weeks, and 14 days were given for recovery before further investigation. Whilst Lee et al (2013) [117] were able to induce radiotherapy resistance using this technique in each of their cell lines, we were only able to do so with one of our cell lines. The failure to establish a radiotherapy resistant phenotype in BICR18 cells may have related to the radiation regime utilised or the nature of the cell line itself. The lack of EGFR expression in the BICR18 cells used may have been a factor.

Cell lines are susceptible to genetic alterations over time that may not necessarily relate to radiation. For this reason the use of a passage matched parental cell line is useful as a control [120]. Whilst this allows for comparisons between parental and treated sub line cells, it does not account for potential changes in global gene expression that may occur with age[121]. This is only possible if comparisons are made with the original early passage cell line. BICR18 cells have previously been reported to express EGFR[122], the lack of its expression within our cells could be a result of genetic alterations over time. In an attempt to avoid missing further genetic alterations that may have impacted

on the radio sensitivity of BICR18 and SQ20B cells, both early and late passage parental cells were tested. The relative radio-sensitivity of these cells was compared (**Figure 2-10c and 2-11c**). There was no difference in radio-sensitivity between early and late stage parental cells; a detected difference between the parental and treated cells was therefore likely a result of repeated irradiation.

It is important to note that any conclusions based on irradiated models developed in vitro using these methods may be cell line specific, giving limited clinical applicability. This study is clearly limited by this fact. Nonetheless published data has suggested common survival advantages amongst radiotherapy resistant sub-cell lines [118]. These include increased proliferative capacity, modified cell cycle distribution, increased ability to repair DNA damage and reduced oxidative stress and apoptosis in response to radiation [118]. Although each of these factors may be measured individually, CSA is considered the gold standard test for determining radiosensitivity and is related to each of these factors [123]. In the presented study, CSAs confirmed 55GySQ20B cells to be less radiosensitive than parental cells, suggesting the presence of one or more of these survival advantages in the repeatedly irradiated cell line 55GySQ20B.

#### 2.5.2 IGF-1R and EGFR in Radiotherapy Resistant Laryngeal Cancer Cells

Western blots comparing EGFR, IGF-1R and other communal downstream receptor targets showed that repeated irradiation resulted in up regulation of total IGF-1R in both cell lines and EGFR in SQ20B. Whilst investigating possible causes of enhanced AKT activation we also found down regulation of PTEN in radiotherapy resistant laryngeal cancer subline 55GySQ20B. PTEN is a tumour suppressor gene whose expression is suppressed in up to 30% of HNSCCs [124]. Snietura et al (2012) [125] have previously

reported an association between low PTEN expression detected by immunohistochemistry and resistance to post operative radiotherapy in HNSCC.

The results shown here suggest that repeated irradiation had the potential to up-regulate IGF-1R and EGFR and induce a radiotherapy resistant phenotype in laryngeal cancer cell lines. It was unclear if up regulation of IGF-1R and EGFR was a bystander consequence of radiotherapy or the mechanism underlying relative radiotherapy resistance; it could have involved both. Although, the failure of BI836845 alone to radiosensitize either cell line argues against this. Whilst IGF-1R and EGFR overexpression is associated with radiotherapy resistance in HNSCC, with both signalling pathways activated in response to radiation [95], our study was the first to demonstrate up regulation of IGF-1R in cells subjected to repeated irradiation.

The association between high EGFR expression and radiotherapy resistance has been known for over two decades. Sheridan et al (1997) [126] demonstrated a strong association between radiotherapy resistance and high levels of EGFR using HNSCC cell lines derived from primary tumours. Activation of membranous EGFR results in endocytosis and nuclear transfer via importin  $\beta$ -1. Nuclear EGFR has three main features distinguishing it from its membranous counterpart. It can function as a co-transcription factor and co-regulates cyclin D1, inducible nitric oxide synthase, cyclooxygenase-2, aurora kinase A, c-Myc, breast cancer resistant protein and Stat1 [127-133]. Nuclear EGFR enhances cellular proliferation by phosphorylating proliferating cell nuclear antigen. EGFR is also known to enter the nucleus following radiation therapy to repair radiation induced DNA damage, and this function is



inversely related to patient survival in a number of cancers including oropharyngeal cancer [134].

Whilst not all studies have concurred [135] the overwhelming evidence for up regulation of EGFR in radiotherapy resistant HNSCC has led to the development of therapies targeting this receptor [136]. The EGFR monoclonal antibody Cetuximab has shown some treatment benefit in the context of recurrent or metastatic HNSCC, but acquired treatment resistance has limited its use [137].

IGF-1R has been associated with radiotherapy resistance in solid organ tumours, including breast [138] and malignant melanoma [74]. In vitro, murine and in vivo models have demonstrated up regulation of IGF-1R in radiotherapy resistant cancer phenotypes [63,73,74,53]. IGF-1R is thought to play a role in cell proliferation, DNA repair and cellular apoptosis. Higher levels of IGF-1R associate with increased cell proliferation and inhibition of apoptosis [63]. Clinically in tumours with high IGF-1R this could mean that during daily radiotherapy treatments a significant proportion of the dose given is used to control cells re-populated from the previous day. High proliferative tumours therefore have a poorer prognosis; this has previously been described in HNSCC patients [139-141].

The EGFR and IGF-1R receptors are thought to cross-communicate, whereby inhibition of one receptor can result in up regulation or activation of the other [51,142,50]. IGF-1R up regulation has been associated with resistance to EGFR inhibitors in Ewing sarcoma [143]. Given their communal downstream signalling targets (**Figure 2-1a**) it is logical

to hypothesize that a similar mechanism may contribute to resistance to treatments targeting EGFR in HNSCC.

### 2.5.3 Testing IGF-1R and EGFR Inhibition to Overcome Radiotherapy Resistance in Laryngeal Cancer Cells

Afatinib and BI836845 demonstrated both receptor and downstream target inhibition in BICR18 and SQ20B parental and irradiated cells. In parental BIRC18 cells there was no significant treatment benefit from BI836845 or Afatinib separately or in combination with and without radiotherapy. In 55GyBICR18 cells, which demonstrated up regulation of IGF-1R, combined EGFR and IGF-1R inhibition appeared to induce a significant reduction in cell survival when compared to no treatment, the individual drugs being ineffective (**Figure 2-12b**). However, 55GyBICR18 cells were only modestly radioresistant, and were not radiosensitized by IGF-1R and/or EGFR inhibition.

In parental SQ20B cells, Afatinib (100nM) alone and in combination with BI836845 (100nM) limited cell survival (**Figure 2-7**). BI836845 alone did not effect cell survival or radiosensitize cells. In the absence of radiotherapy, Afatinib (100nM) and Afatinib with BI836845 (100nM) reduced colony survival by 34.6% and 66.5% respectively. The effect of combined treatment was significantly greater ( $p=0.007$ ), although was not significantly different from the effect of Afatinib alone at the higher dose of 1000nM. The results were not replicated in radiosensitivity assays, where Afatinib (100nM) alone seemed to be as effective as Afatinib with BI836845 (100nM) in radio sensitizing cells (**Figure 2-7b**).

The repeatedly irradiated 55GySQ20B cells were relatively radio-resistant (**Figure 2-11b**), and here, combined Afatinib and BI836845 (100nM) treatment appeared to be more effective than treatment with Afatinib (100nM) alone at inhibiting basal cell survival. The treatment effect was greater in irradiated cells with 52.4% survival in SQ20B cells vs. 22.9% survival in 55GySQ20B cells ( $p=0.0001$ ) (**Figure 2-13a**). Whilst baseline colony survival was affected by combined drug treatment, the results were not replicated in the presence of radiotherapy. In this cell line, Afatinib enhanced radiosensitivity but BI836845 with and without Afatinib did not (**Figure 2-13b**).

These results suggest that combined drug treatment with Afatinib and BI836845 was more effective at suppressing basal cell survival in radiotherapy resistant cells with up regulation of IGF-1R and EGFR. However, despite affecting baseline colony survival, both treatments alone and in combination were unable to radio-sensitize either cancer cell line. This suggests that the IGF-1R up regulation induced by irradiation was not a driver of radioresistance.

There is evidence that IGF-1R inhibition can increase radiation sensitivity in some HNSCC cell lines [95] and augment the effects of radiotherapy in other solid organ tumours [144,74]. Our results did not support this, as BI836845 alone had no effect on cell survival or radiosensitivity in either of the two cell lines tested here. This could be explained by the fact that BI836845 is an IGF-ligand antibody instead of an IGF-1R monoclonal antibody (previously shown to induce radiosensitivity as described by Raju et al (2015) [95]). There was clear evidence of receptor cross talk between EGFR and IGF-1R in the SQ20B cell line. EGF ligand was shown to activate IGF-1R whilst IGF ligand did not have this effect on EGFR. Our results showed that EGF ligand

stimulation was associated with phosphorylation of IGF-1R, which was not inhibited by the use of BI836845 (**Figure 2-14**), suggesting an IGF ligand independent effect. In the absence of EGF ligand stimulation BI836845 was effective at inhibiting IGF-1R phosphorylation. The failure of this drug to induce radio sensitivity in the SQ20B cells may have been a result of co-stimulation of IGF-1R via EGFR (or one of its family members) without the need for IGF ligand, thus bypassing the effects of BI836845.

In BICR18 cells where EGFR was absent, BI836845 had no effect on cell survival or response to radiotherapy, despite confirmation of target inhibition using western blots. In this cell line an alternative mechanism may have bypassed the effects of the drug via the RAS/ERK pathway. Western blots showed that BI836845 inhibited phosphorylation of AKT but not ERK, which may have allowed for ongoing cell proliferation and survival along this pathway and explained lack of response to this drug.

The IGF-1R pathway is thought to support resistance to EGFR targeted treatments and so combined receptor inhibition is expected to provide better results than single agent treatment [145,146]. Combined EGFR and IGF-1R inhibition has shown benefit in overcoming resistance to radiotherapy in prostate cancer [147]. In vitro and animal studies[94] have shown that combined IGF-1R and EGFR inhibition causes a more significant reduction in cell survival than either agent alone in HNSCC cells [148,149]. IGF-1R inhibition using a monoclonal antibody in combination with Gefitinib (EGFR inhibitor) has been found to overcome initial treatment resistance and prevent tumour recurrence in mice whilst single agent treatment does not [94]. However, in clinical studies of IGF-1R antibody for treatment refractory HNSCC, there has been less success [150].

Barnes et al (2007) [148] and Slomiany et al (2007) [149] both reported improved efficacy in reducing HNSCC cell survival when inhibiting the EGFR and IGF-1R pathways. The current study also found combined EGFR and IGF-1R inhibition had a greater impact on basal cell survival than either agent alone, with a greater effect in SQ20B cells with up regulation of IGF-1R. This suggests that irradiation could induce greater dependence on the IGF and EGF axes.

Recently Raju et al (2015) [95] compared the effects of Cetuximab and IGF-1R monoclonal antibody IMC-A12 on HNSCC cell viability and radiation sensitivity using clonogenic survival assays. They found IMC-A12 did not enhance the effects of cetuximab in the presence of radiation but did increase radiotherapy sensitivity in one of the six cell lines tested. Their study reported no treatment benefit from combined IGF-1R and EGFR inhibition, suggesting that other RTKs and/or their downstream effector proteins may have compensated for the loss of EGFR and IGF-1R activity. In contrast, the present study found reduced cell survival with the use of EGFR receptor inhibition and combined EGFR and IGF-1R inhibition in repeatedly irradiated cells.

The clinical impact of combined EGFR and IGF-1R inhibition remains largely untested. On-going trials of BIIB022 (IGF-monoclonal antibody) and IMC-A12 in relapsed/refractory and advanced solid tumours [96,97], and a randomized trial of preoperative cetuximab and/or IMC-A12 in patients with HNSCC [98] are likely to provide more information on the clinical applicability of targeting these receptors.

#### 2.5.4 Study Limitations

This study revealed IGF-1R up regulation in irradiated LSCC cells and suppression of cell survival by dual IGF-1R/EGFR inhibition in repeatedly irradiated cells. Whilst the results of this study are promising, they should be interpreted with caution. This is the first study to report on the efficacy of BI836845 in HNSCC and utilised only two cell lines. Whilst this has advantages in that it allows for detailed analysis of the cell lines and potential impact for this drug, it does limit the generality of the results. One advantage of this study over others is that one cell line expressed EGFR whilst the other did not; this is relatively uncommon in HNSCC. This allowed comparison of the effects of single and combined drug inhibition in both cell types, and used early and late passage parental cells as controls; to our knowledge this has not been done before.

We were unable to geno-typically verify SQ20B cells, and BICR18 cells displayed different characteristics from those previously described in the literature [151,122]. This meant that genetic variation may have occurred over time and the tested cells might not have reflected the original laryngeal tumour they were cultured from. Primary cell culture from current patients would be useful to overcome this.

Stanton and colleagues (1994) demonstrated expression of EGFR in BICR18 cells using radio-iodinated ligand binding [122]. In the present study, western blotting was used to detect EGFR in the BICR18 cell line. The inability to detect EGFR in BICR18 cells in this study may have reflected the lower sensitivity of western blotting when compared to radio-iodinated ligand binding to detect EGFR. A different EGFR antibody or alternative technique for detection might have provided different results. It is also

possible that the ligand used by Stanton and colleagues may have bound to something other than EGFR, providing a false positive result.

Interestingly, Stanton and colleagues cultured BICR18 cells on lethally irradiated NIH3T3 feeder cell layers; this was not performed in the present study. The relative difference in EGFR expression and growth in the absence of lethally irradiated NIH3T3 feeder cell layers may have contributed to or represented an epigenetic change in the cell line, a phenomenon known to occur during cell culture over a prolonged time[152].

More recently Li et al (2014) described BICR18 as having mutations in the PI3K signalling pathway[153]. It is plausible that this might have led to decreased reliance on RTK signalling and hence down regulation of EGFR or reliance on signalling via an alternative RTK.

Further analyses to detect EGFR in the BICR18 cell line might have included quantitative real time polymerase chain reaction (qRT-PCR) for EGFR RNA. This highly sensitive technique would have allowed for the detection of low levels of EGFR otherwise undetected by western blotting. An alternative approach might have been to treat cells with 5-azacitidine (a chemical analogue of the nucleoside cytosine), to demethylate genomic DNA to determine whether this would allow re-expression of an epigenetically silenced EGFR gene, followed by repeat western blotting.

Overall, the inability to detect EGFR in the BICR18 cell line (despite cell line verification using STR) may have represented an acquired genetic alteration in the cell

line over time, changes acquired during cell culture, or reflected a lack of sensitivity during the detection process.

This study was also limited by the principles of cell culture, since conventional 2D cell culture models do not reflect the true growth conditions or microenvironment of HNSCCs. Given the heterogeneous nature of HNSCCs there are likely to be a wide range of cell types present within the same tumour with EGFR and IGF-1R expression varying between cells [154]. This issue may be addressed by future studies utilising 3D cell culture incorporating components of the tumour microenvironment, animal models and clinical trials.

Drug testing on all cells was performed using triplicate technical replicates but due to time constraints not all the results were repeated. The effects of drug treatment on irradiated cells should ideally be performed three times to confirm the reliability of results.

#### 2.5.6 Recommendations for On-going Research

Further evaluation of the relationship between IGF-1R and EGFR expression and radiotherapy resistance in laryngeal cancer should assess tumour biopsies prior to and following radiotherapy. This will allow levels to be compared between patients that do and do not respond to radiotherapy to see if IGF-1R levels predict treatment response. This would provide in-vivo evidence for the association of IGF-1R with radiotherapy resistance in LSCC. The key issue of whether radioresistance could then be overcome by using IGF-1R or EGFR inhibitors however would still remain.



Combined IGF-1R and EGFR inhibition has shown benefit in limiting the survival of LSSCC cells as evidenced by the present study (**Figure 2-12b, 2-13a**). This combination could have a role as neo-adjuvant chemotherapy in the clinical setting, by reducing tumour bulk prior to radical treatment. Preclinical in vivo and clinical trials will be needed to evaluate this potential.

IGF-1R and EGFR combined inhibition has failed to result in increased radio-sensitization of HNSCCs. A better appreciation of the interaction between these two receptors and other potential contributing RTKs is necessary. Microarray analysis comparing parental and irradiated or relatively radio-resistant cell lines may provide further information about other contributing factors.

## **2.6 Conclusions:**

Radiotherapy is frequently offered as first line treatment for early LSCC. At present we are unable to predict the patients most likely to benefit from this treatment. This study aimed to evaluate the role of IGF-1R in radiotherapy resistant laryngeal cancer cells. Using repeated irradiation it was possible to induce a radiotherapy resistant phenotype in one of two cell lines, which expressed higher levels of IGF-1R. This may be clinically relevant given data presented in the previous chapter, in which tumours with higher levels of IGF-1R appeared to be more resistant to radiotherapy. Furthermore, evidence of receptor ‘cross-talk’ was identified between EGFR and IGF-1R; this could contribute to treatment resistance to EGFR inhibitors in HNSCC.

In summary, combined EGFR and IGF-1R inhibition limited cell survival in repeatedly irradiated LSCC cell lines, suggesting induced dependence. However, there was no

enhancement of the effects of radiotherapy. There is a need for further investigation into the interaction between these two receptors and other RTKs that might play a role in radiotherapy resistant HNSCC. Future studies should focus on the analysis of patient tissue pre and post radiotherapy and the evaluation of drugs targeting alternative components of the IGF-1R/EGFR axes, and additional signalling cascades in an attempt to develop clinically relevant strategies to radiosensitize.

**Chapter 3: Current and Future Techniques for Human Papilloma Virus (HPV)**  
**Testing in Oropharyngeal Squamous Cell Carcinoma**

**This chapter is based on a recently published peer review article:**

Qureishi A, Mawby T, Fraser L, Shah KA, Møller H, Winter S (2017) Current and Future Techniques for human papilloma virus (HPV) Testing in Oropharyngeal Squamous Cell Carcinoma.

Eur Arch Otorhinolaryngol. doi: 10.1007/s00405-017-4503-1. [Epub ahead of print]

**Abstract:**

Despite a reduction in smoking and alcohol consumption, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) is rising. This is attributed to human papilloma virus (HPV) infection and screening for HPV is now recommended in all cases of OPSCC. Despite a variety of clinically available tests and new non-invasive test strategies there is no consensus on which technique is best. This review reports on current techniques for HPV detection in OPSCC and the clinical applicability of emerging techniques.

Literature searches of Medline, Embase and clinicaltrials.gov using the search terms 'head and neck neoplasms', 'squamous cell carcinoma' and 'HPV testing' were performed. 45 studies were identified and included.

p16 immunohistochemistry (IHC), HPV DNA in-situ hybridization (ISH) and HPV polymerase chain reaction (PCR) are the commonest tests to determine HPV status. p16 IHC and HPV DNA PCR are highly sensitive whilst HPV DNA ISH is more specific, these techniques conventionally utilize surgical biopsies. New tests using PCR to screen fine needle aspirates, saliva, brush cytology and serum for HPV are promising but have variable sensitivity and specificity. These non-invasive samples avoid the morbidity of surgical biopsies and need for tissue blocks; their clinical role in screening and surveillance remains largely untested. Further work is needed to validate these tests and define their role.

### **3.1 Background:**

Despite a reduction in smoking and alcohol consumption, the UK incidence of oropharyngeal squamous cell carcinoma (OPSCC) doubled from 1990 to 2006 and again from 2006 to 2010 [155]. This increase is mirrored worldwide and is attributed to human papilloma virus (HPV) associated OPSCC. HPV positive (HPV+ve) OPSCC is set to overtake the incidence of HPV associated cervical cancer by 2020 and is a major health epidemic in the western world[21]. HPV is a common infection with a prevalence of 7%. High-risk sub-types e.g. 16 and 18 are responsible for HPV +ve OPSCC and are present in 1% of the population [156].

Ang et al 2010 [157] demonstrated that the prognosis and response to treatment for HPV negative (HPV-ve) OPSCC was different to HPV+ve disease. HPV+ve OPSCC affects younger non-smoking individuals with high-risk sexual practices. Nodal metastases are more frequent with patients tending to have a higher N stage at presentation [158]. The presence of HPV in OPSCC can predict prognosis, guide treatment decisions and determine suitability for entry into de-escalation clinical trials aimed at improving long term quality of life [24]. Local-regional treatment failure and 5 year survival is reported at 13% and 82% for HPV+ve OPSCC whilst it is 42% and 35% in HPV negative (-ve) disease [23].

HPV is the only utilised biomarker for patients with head and neck squamous cell carcinoma (HNSCC) but is only clinically useful for patients with OPSCC. HPV testing on surgically obtained biopsies is considered the standard of care for patients with OPSCC and is recommended by NICE [159]. Despite this UK and US head and neck cancer departments only test HPV in 79% and 67% of cases routinely [160], the main

reasons for not testing are cost, lack of clinical relevance and time constraints [161]. Concerns over individual clinician knowledge regarding indications for HPV testing have been raised with one US centre reporting clinically inappropriate requests for HPV testing in 77% of cases [162].

The need for HPV testing when treating patients with OPSCC is well established. The ideal test would be non-invasive, economical, of low complexity and easily incorporated into routine clinical practise. Such a test could form part of screening and monitoring programmes for OPSCC and shorten time to diagnosis and treatment, improve patient satisfaction and overall morbidity and mortality. At present there is no consensus on HPV testing in OPSCC with various methods described [158]. Clinical features including cancer stage, site, tumour differentiation, presence of cystic metastasis, age, race and smoking status do not accurately predict HPV status and so specific clinical testing is required [163].

### **3.2 Aims:**

This review aims to help practising head and neck surgeons understand the techniques available for detecting HPV in OPSCC (**Table 3-1**), their relative advantages, disadvantages (**Table 3-2**) and clinical applicability to non-invasive ‘liquid biopsies’ (**Table 3-3**). It is hoped this information may help guide clinical decisions for future testing of HPV in OPSCC and increase compliance with national guidelines.

### **3.3 Methods:**

A search of available literature was conducted on 28 August 2016 using Medline, Embase and clinicaltrials.gov from 1996-2016. Search terms were combinations of

‘head and neck neoplasms’, ‘squamous cell carcinoma’ and ‘HPV testing’.

A total of 343 articles were identified, after abstract, article and reference review a total of 45 studies were included in this review.

### **3.4 Results:**

The most commonly utilised techniques for HPV testing in OPSCC are p16 immunohistochemistry (IHC) (recommended by NICE [159]), polymerase chain reaction (PCR) and DNA in situ hybridisation (ISH) (although other techniques have been described) their sensitivity and specificity is described in **Table 3-4**. Besides sensitivity and specificity each test differs in availability, expertise required, cost and time which has resulted in uncertainty as to which test or combination of tests should form the clinical standard [159].

HPV testing is typically performed on formalin-fixed paraffin embedded (FFPE) tissue blocks although some authors have described their use on fine needle aspirates (FNA), saliva, brush cytology and serum/plasma.

#### ***3.4.1 Routine Histology***

HPV+ve OPSCC has a characteristic appearance on routine haematoxylin and eosin staining. Arising from tonsillar crypt epithelium the cells are small with indistinct borders, generally round/oval nuclei, high levels of mitosis, and variable necrosis. Keratinisation is often focal and tumours predominantly non-keratinising [158,164]. HPV+ve OPSCC's tend to invade as sheets, lobules or ribbons of cells in the absence of strong desmoplastic stromal reaction [164]. Some authors have described these

characteristic features as 99% specific for HPV infection [165]. A small proportion of keratinizing OPSCC's however, are HPV+ve and so histology alone cannot be relied upon to make an accurate diagnosis of HPV infection.

#### 3.4.2 Southern Blotting

Southern blotting is an established technique for detecting HPV DNA, enzymes are used to 'digest' target DNA from fresh frozen samples before being separated using gel electrophoresis and transferred to a membrane. Target proteins are hybridized with cloned HPV genomic probes before detection. Technical complexities and an inability to utilise FFPE tissue samples make this technique impractical in the clinical setting [166].

#### 3.4.3 p16 Immunohistochemistry (IHC)

IHC typically utilizes antibody mediated staining of tumour samples taken from FFPE tissue blocks to allow a pathologist to determine the relative abundance and intensity of a protein. The oncogenic HPV protein E7 binds and inactivates retinoblastoma tumour suppressor gene (pRb) resulting in decreased pRb and Cyclin D1 and increased p16 expression [167]. p16 is considered a surrogate marker of transcriptionally active HPV infection. Although interpretation of IHC is subjective only strong staining is taken to indicate positivity. Studies have reported 97% agreement between pathologists in interpreting results [168].

p16 expression is considered by some as the most reliable prognostic marker for OPSCC and is an independent predictor of survival although this is not always the case [169]. p16 has high sensitivity for HPV (94%) [170], low cost and is readily available



and interpretable (when signal intensity is high). These desirable features have led to p16 IHC being commonly adopted as a stand alone technique for HPV detection in clinical and research settings and is currently recommended for all cases of OPSCC in the UK.

Two arguments have emerged against isolated p16 testing for determining HPV status in OPSCC. The first is limited reliability of interpretation when staining is weak or equivocal (<5% of cases [168]). Current UK guidelines recommend >70% staining to confirm p16 positivity[159]. When staining is lower a supplementary test is often required which may not necessarily be available in all laboratories. Whilst there is evidence to support HPV positivity with diffuse relatively weak p16 staining the associated subjectivity makes the test unreliable in this context [171].

The second relates to the specificity of p16 testing, some authors have reported up to 20% of p16-positive OPSCC's to be HPV-ve [169]. In this context it is thought that p16 is elevated by non-virus related alterations. A recent meta-analysis of 25 studies has confirmed a statistically significant number of false positives with p16 IHC alone when compared to p16 IHC and an additional test for HPV DNA/RNA [169]. The authors advocated that p16 testing should be supplemented with an additional test (either PCR or DNA/RNA ISH) to confirm the presence of HPV.

If HPV status is required to make treatment decisions in the context of de-escalation treatment or new molecularly targeted therapies, in the worst-case scenario p16 alone could result in 1 in 5 patients receiving clinically inadequate treatment. With the availability of additional tests to confirm HPV DNA these risks can be avoided.

#### 3.4.4 DNA in-situ Hybridisation (ISH)

In situ hybridisation is a technique that amplifies signals on molecular probes, which specifically bind to HPV DNA. There are readily available probes for the detection of all known HPV strains. FFPE tissue can be analysed for high-risk HPV sub-types using an automated platform and viewed with conventional light microscopy. This technique is frequently available in modern pathology laboratories. DNA ISH allows for differential detection of nuclear and diffuse staining patterns providing a histological context for HPV within tumour cells.

DNA ISH is an excellent clinically available test for confirming the presence of integrated HPV in OPSCC. Studies have shown 99% concordance between HPV detection in E6/E7 mRNA and HPV DNA ISH [172].

DNA ISH could be the ‘clinically viable gold standard’ test for HPV detection in OPSCC given its superior specificity of 88% compared to 82% with p16 IHC[170]. The test however has a low sensitivity particularly when HPV DNA copy numbers in tumour tissue are low. DNA ISH also has a lower sensitivity of 88% compared to 94% in p16 IHC [170]. Furthermore as DNA ISH detects DNA and not mRNA it does not confirm transcriptional activity, it is more expensive than p16 IHC and has an 11% reported inter-observer variability [168].

#### 3.4.5 RNA in-situ Hybridisation (ISH)

RNA ISH is superior to DNA ISH as it confirms the presence of transcriptionally active HPV. Even when copy numbers are low translocation amplifies the signal making RNA detection easier [172]. RNA ISH is promising as it identifies HPV driven cancers

in a single test, avoids risks of contamination (as seen in PCR), utilises FFPE tissue samples and has easily interpretable results[173]. RNA ISH is more sensitive and specific than DNA ISH in detecting HPV in OPSCC (97% and 93% vs. 88% and 88%) and correlates strongly with p16 expression [170].

RNA ISH testing however is not available on an automated platform and is considered too laborious for routine use [174]. This could explain why there are relatively few studies of RNA ISH for HPV in OPSCC and why the technique is not yet approved for clinical use.

#### 3.4.6 Polymerase Chain Reaction (PCR)

PCR is a technique used to amplify signal from mRNA or DNA by several orders of magnitude to detect target DNA using pre-specified primers. It is the most widely utilised tool for detecting and genotyping HPV.

Sustained and persistent high-risk HPV E6/E7 viral oncogene expression drives the HPV-associated malignant phenotype. Detection of E6/E7 mRNA by reverse transcriptase PCR of E6/7 mRNA transcripts indicates the presence of transcriptionally active HPV, the “gold standard” for HPV testing [175]. Until recently this required the use of fresh frozen tissue but is now possible in FFPE samples, currently this is almost exclusively used in a research setting [172].

PCR to detect HPV DNA in FFPE is relatively easy to conduct, highly sensitive, cost effective and widely available. There are a variety of commercially available assays, with varying performance [176]. The highly sensitive nature of the test means that

previously amplified material can contaminate negative specimens and it is not always possible to tell if the DNA detected relates to the tumour cells or surrounding normal tissue. The reported sensitivity and specificity for this test is 97% and 87%, respectively [170].

Besides its use in tissue samples, PCR has been used to assess the presence of HPV in saliva, fine needle aspirates and serum plasma.

#### 3.4.7 Combination Testing

Combining tests for HPV may improve the accuracy of results and two-step diagnostic algorithms have been described that include p16 IHC followed by PCR for HPV DNA or p16 IHC followed by HPV DNA ISH. As an initial screen p16 positivity (which has high sensitivity) indicates the presence and transcriptional activity of HPV which can be confirmed as originating from the tumour by DNA ISH (greater specificity) [166]. The first algorithm has been validated in FPPE tissue against the gold standard of PCR for E6/7 mRNA whilst the second more practical combination has been used in large epidemiology studies and clinical trials and is validated as a prognostic marker.

To overcome conflicting results between two tests Thavaraj et al [168] described a three-step technique of p16 IHC, HPV ISH and PCR alongside consensus reporting for ISH to improve molecular classification of tumours. In this algorithm PCR was used in cases where the sample was p16 positive but DNA ISH negative. PCR confirmed the presence of HPV DNA allowing 98% of samples to be described as HPV+ve or -ve.

Schache et al [170,177] compared the relative diagnostic accuracy of single and combined HPV tests on FFPE tissue in 108 cases of OPSCC. They compared p16 IHC and HPV ISH, p16 IHC and DNA PCR and p16 IHC and RNA ISH to each test individually reporting combined test sensitivities of 89%, 97% and 93% and specificities of 90%, 95% and 100%. Combined tests demonstrated improved diagnostic accuracy for HPV detection when compared to each test in isolation.

Whilst combination testing may improve diagnostic accuracy whereby one test compensates for the weaknesses of another it is resource intense and a single test would be preferable. In the same study Schache et al [170,177] showed that RNAscope (RNA ISH) had 94% sensitivity and 100% specificity, raising the possibility for single test algorithms to eventually provide the solution to this diagnostic problem.

#### 3.4.8 Fine Needle Aspiration (FNA)

Fine needle aspirates are routinely utilised in HNSCC diagnosis and in some patients represent the only available diagnostic material. DNA ISH, PCR and p16 IHC can all be used to determine the HPV status from cancer cells obtained by FNA when cellblocks are prepared from aspirated material. At present there is no consensus on HPV testing of FNA samples.

Despite concerns regarding potential subjective interpretation particularly with low cell numbers, HPV DNA ISH on FNAs constructed as cellblocks from patients with OPSCC may prove useful. Begum et al [178] detected HPV16 in 53% of metastatic OPSCCs but not in FNAs from other head and neck tumour sites.

Others have applied these detection strategies to ethanol fixed smears of fine needle aspirates, avoiding the need for cell blocks[179]. Before clinical implementation prospective large-scale diagnostic accuracy studies for DNA ISH on FNA material are needed.

Determining the proportion of p16 staining on FNA specimens can be difficult and is confounded by the fact that 50% of benign branchial cysts are often p16 positive. This is a practical dilemma as nodal metastases from OPSCC's and branchial cysts occur in a similar age group, contain squamous epithelium and when associated with inflammation, cytology alone cannot confirm or exclude malignancy. In these cases p16 status is unhelpful, as it does not predict prognosis or malignant potential.

Recommended thresholds for 70% p16 staining may not apply to FNAs, instead lower thresholds of 10% give reported sensitivity and specificity of 94% and 75% [180]. There is a need for further research to validate the use of p16 IHC on FNA samples in the context of OPSCC.

Liquid based PCR assays originally developed for cervical cancer may also prove useful on FNA samples; these include the Hybrid Capture II, Cervista HPV HR, Roche Cobas and APTIMA tests. PCR on liquid samples avoids the need to create tissue blocks but the tests require clinical validation. Isolated tests of HPV DNA PCR on FNAs have reported 94.7% sensitivity and 100% specificity [181].

#### 3.4.9 Brush Cytology

HPV DNA PCR on brush cytology specimens is the clinical standard for HPV DNA detection in cervical cancer, however this is not the case in OPSCC [182]. Despite high sensitivity, low cost and availability it has not gained popularity in OPSCC. Direct cytological examination of brushed material is of limited value as small tumours are confined to tonsillar crypts and may not be sampled. Also, a surface “in-situ” component is often lacking, reducing the utility of morphological assessment in such samples.

Both p16 IHC and PCR have been successfully used to detect HPV using brush cytology. Cytological assessments may have higher levels of sensitivity and specificity for HPV detection when compared to similar tests on FFPE samples and can provide results in just a few hours.

Brogile et al [183] compared the results of PCR and p16 on brush cytology specimens from patients with OPSCC to conventional p16/PCR tests on FFPE and reported sensitivity and specificity of 83% and 94%. Larger studies by Dona et al [184] of 164 brush cytology samples and Marques et al [185] showed 90.04% and 94.7% concordance between cytochemical and FFPE assessment for HPV. The latter study however reported inadequate sampling using brushings in 45.7% of individuals; these results may have been affected by bias in the study design.

Linxweiler et al [186] compared combined PCR for p16INK4a (a tumour suppressor associated with high-risk HPV) and Ki67 (a marker of high cell turnover) on cytological specimens to PCR on FFPE in 20 cases. Their results demonstrated 95% concordance

and showed the ability to recognise details of cellular morphology including keratinization. Overall liquid based cytology had a diagnostic sensitivity of 98% and 100% for diagnosing HNSCC. The results of cytological assessment using brushings are promising for detecting HPV and is an area requiring greater attention.

#### 3.4.10 Saliva

Testing bodily fluids from an infected area for the presence of HPV is an established concept. Recent meta-analyses of urinary tests as opposed to cervical smears for cervical cancer screening have reported sensitivity and specificity of 73% and 98% [187].

A saliva test that is non-invasive, relatively inexpensive, without side effects and taken at the point of first patient contact could allow for the HPV status of patients to be established much earlier. If proven successful, salivary testing for HPV could form a routine part of screening and risk stratification in patients suspected of OPSCC. It may also have a role in monitoring patients post treatment for signs of recurrence. Salivary testing would avoid the need to create tissue blocks required for current techniques that are costly and time consuming. One study has estimated a potential cost saving of \$365 per patient [188]. The potential for saliva testing for HPV has led to increased media attention and speculation on its use in national screening programmes [189].

HPV is readily detected in the saliva of individuals and saliva testing has been used extensively as part of screening programmes in non-cancer patients. Levels appear to be higher in those that smoke, drink alcohol in excess and engage in high-risk sexual activities [190].



Saliva testing for HPV infection in OPSCC patients has been successfully trialled in HNSCC patients; high-risk HPV types can reliably be detected in the saliva of patients with HNSCC and could be used to predict the HPV status of OPSCC.

The sensitivity of salivary testing has been questioned with some authors reporting an inability to detect HPV from oral rinse specimens in patients with known HPV+ve OPSCC[191]. It is thought that the origin of HPV +ve cells may effect the specificity of tests on saliva as HPV could be detected from infection within non-tumour tissue or be difficult to detect with lower tumour stage [166]. Wang et al [192] showed that saliva was inferior to plasma when testing for HPV in patients with OPSCC; they detected HPV in 40% of their salivary specimens and 86% of their plasma specimens.

There are a number of commercially available liquid assay tests that could be used to test saliva but there is a need for their validation in clinical practice. These tests have shown promise with FNA samples [193] but need to be assessed with saliva. Kerr et al [194] recently demonstrated 100% sensitivity and 86% specificity when using the PCR Roche Cobas 4800 test in comparison to HPV DNA ISH. Whilst Ahn et al [195] reported salivary HPV testing in combination with plasma testing could reliably predict HPV status and recurrence in OPSCC with >90% specificity and 69.5% sensitivity.

Saliva testing for HPV is a promising area for research and may have a role in diagnosing and monitoring patients with OPSCC. There is a need for prospective adequately powered diagnostic accuracy studies comparing the efficacy of salivary tests to the clinical standards of p16 IHC, DNA ISH and HPV DNA PCR on FFPE in patients with OPSCC before salivary testing forms a part of routine clinical practise.

#### 3.4.11 Serum Antibodies / DNA

Serological assays have the potential to identify HPV through antibody detection, however infections outside the primary OPSCC could influence the result. The findings of a large study comparing serum samples from 292 HNSCC patients to 1568 matched controls contradicted this hypothesis. The group used ELISA to detect L1 and L2 proteins (associated with high-risk HPV types 16, 18 and 33) amongst healthy individuals and those with head and neck cancer and found these to be largely attributable to infection within tumour. The odds ratio for detecting HPV for HPV+ve tumours was 37.5 compared to 2.1 for HPV-ve tumours [196].

Anti E6/E7 antibodies have been detected in the serum of HNSCC patients, these findings are thought to represent active HPV driven transformation of HNSCC as their detection in non HNSCC patients is rare [166]. In fact if evidence from serum testing for cervical cancer is to be applied to OPSCC, anti-E6/7 antibody detection post treatment may be a useful prognostic marker.

For nasopharyngeal carcinoma the presence of plasma Epstein-Barr Virus (EBV) DNA is associated with relapse or persistent tumour and can be measured as part of surveillance, similar principles could be applied to HPV+ve OPSCC. Although large prospective trials and optimization is needed, HPV DNA has successfully been detected by PCR in the plasma of patients with HPV +ve HNSCC.

### **3.5 Discussion and Conclusions:**

HPV testing for OPSCC is complex with various techniques described using FFPE tissue and ‘liquid biopsies’ e.g. saliva, brush cytology, FNA and serum.

Current evidence suggests that conventional p16 IHC is useful as a screening tool for determining HPV status in FFPE samples from tumour biopsies and should be considered the first line test in all cases of OPSCC. Given the findings of a recent meta-analysis there is evidence to support additional testing using a technique with greater specificity e.g. HPV DNA ISH or PCR [169].

There is a need for a single modality test to increase widespread adoption and limit costs. Further validation of RNA ISH may prove useful in providing this desirable solution.

Non-invasive testing for HPV in cervical cancer is now a reality and it is hoped that the techniques utilised here may be transferred to HPV testing in OPSCC. Whilst brush cytology, saliva and serum have proven effective in determining HPV status there are no large prospective clinical trials assessing their clinical effectiveness. These techniques should be assessed independently and in combination for their clinical effectiveness at determining HPV in OPSCC to avoid the need for invasive tissue biopsies and creation of tissue blocks. Clinical validation and optimisation of these tests may lead to ‘liquid biopsies’ being useful in screening, diagnosis and monitoring of HPV+ve OPSCC.

**Table 3-1:** Current techniques for HPV Testing in OPSCC and the tissue samples to which they have been applied.

Diagnostic Technique	Diagnostic Tissue Source				
	Conventional Biopsy	Liquid Biopsies			
	Tumour Tissue	Fine Needle Aspirate	OPSCC Brush Cytology	Saliva	Serum Plasma
Routine Histology	✓	✗	✗	✗	✗
Southern Blotting	✓	✗	✗	✗	✗
p16 IHC	✓	✓	✓	✗	✗
DNA/RNA ISH	✓	✓	✗	✗	✗
PCR E6/E7 mRNA	✓	✗	✗	✗	✗
PCR HPV DNA	✓	✓	✓	✓	✓
HPV antigen detection	✗	✗	✗	✗	✓

Key:
✓ successfully applied
✗ not applicable

**Table 3-2: HPV Testing for OPSCC – Advantages and Disadvantages of Utilised Methods**

Method	Advantages	Disadvantages
<b>Routine Histology</b>	Universally available diagnostic procedure, where OPSCC have distinctive morphological features  Complements additional testing  Low Cost/No additional equipment required	Small proportion of HPV+ve tumours do not exhibit characteristic features
<b>Southern Blotting</b>	Historically well established and reliable	Technically challenging  Cannot be used on FFPE – clinically irrelevant
<b>p16 IHC</b>	Independent predictor of survival  High sensitivity  Low Cost  Easily incorporated into surgical laboratories  Plenty of supportive evidence  May be used on FNA/Brush cytology specimens avoiding surgical biopsy	Is an indirect measure of HPV transcriptional activity – not suitable for determining suitability to targeted therapies  Lower specificity than ISH/PCR  Subjective interpretation particularly with weak staining
<b>DNA ISH</b>	High specificity  Provides histological context for HPV infection  Easily incorporated into surgical laboratories  May be used on FNA specimens avoiding surgical biopsy	Subjective interpretation (up to 11% inter observer variability)  Low sensitivity when viral load low  More costly than p16 IHC  Indirect measure of HPV transcriptional activity – may not be suitable for targeted therapies
<b>RNA ISH</b>	Confirms HPV transcriptional activity  Amplifies low viral signal  Avoids risk of contamination  Greater sensitivity/specificity compared to DNA ISH  Single test with high sensitivity/specificity matching the research 'gold standard' of reverse transcriptase PCR for E6/7 mRNA	Technically demanding  Not a routinely available test  Not available on automated platform  Limited clinical evidence of efficacy
<b>HPV DNA PCR</b>	High sensitivity  Low Cost	Easily contaminated  Detected DNA may not be from tumour tissue

	Widely available (numerous commercial assays)	May not be suitable for targeted therapies in isolation
	Easily incorporated into surgical laboratories	
	May be used on FNA/Saliva/Brush Cytology/Serum Plasma specimens avoiding surgical biopsy	
<b>Combination Testing</b>	High sensitivity/specificity	Two or three tests required
	Histological/biological context as well as p16 status	Higher cost/time
	Allow for accurate tumour sub-classification	

**Table 3-3: Non-invasive HPV Testing for OPSCC – A Summary of ‘Liquid’ biopsies**

<b>Liquid Sample</b>	<b>Advantages</b>	<b>Disadvantages</b>
<b>FNA</b>	<p>Non-invasive test/avoids anaesthetic</p> <p>Maximizes diagnostic information if only tissue available</p> <p>Cellular samples can be processed to cellular pellets for IHC</p> <p>Diverse testing applicable – PCR/DNA ISH/p16 IHC (further clinical testing/validation may be required)</p>	<p>Sample cellularity heavily dependent on underlying cystic change, which if present may not yield a diagnostic sample</p> <p>p16 IHC unhelpful in equivocal cases as 50% of branchial cysts are p16 positive</p>
<b>Brush Cytology of oropharynx</b>	<p>Non-invasive test/avoids anaesthetic</p> <p>Already utilised extensively for cervical cancer</p> <p>Low Cost</p> <p>High sensitivity</p> <p>May provide additional data on cellular morphology</p>	<p>Some authors report high rates of inadequate sampling (up to 45%)</p> <p>Further clinical testing/validation is required</p>
<b>Saliva</b>	<p>Non-invasive test/avoids anaesthetic</p> <p>Successfully utilised for oral/oropharyngeal HPV detection in non cancer patients</p> <p>Low cost/ avoids need for tissue blocks</p> <p>May have a role in post treatment surveillance</p>	<p>Non-specific – unable to tell if HPV from tumour or normal tissue</p> <p>Some authors have questioned sensitivity – as unable to detect HPV in HPV+VE cases of OPSCC</p> <p>Further clinical testing/validation is required</p>
<b>Serum Plasma</b>	<p>Non-invasive test/avoids anaesthetic</p> <p>Avoids need for tissue blocks</p> <p>May be superior to saliva in detecting HPV in HPV+ve OPSCC</p> <p>May have a role in post treatment surveillance</p>	<p>Non-specific – unable to tell if HPV from tumour or normal tissue</p> <p>Further clinical testing/validation is required</p>

**Table 3-4:** Reported Sensitivity and Specificity of HPV Testing in OPSCC (Schache et al 2011 in 108 patients with OPSCC[170]).

<b>Diagnostic Test</b>	<b>Sensitivity</b>	<b>Specificity</b>
<b>p16 IHC</b>	94%	82%
<b>HPV DNA ISH</b>	88%	88%
<b>HPV DNA PCR</b>	97%	87%
<b>DNA PCR + p16 IHC</b>	97%	94%
<b>p16 IHC + DNA ISH</b>	88%	90%



## **Chapter 4: Saliva Testing for HPV in Oropharyngeal Squamous Cell Carcinoma (OPSCC): A Diagnostic Accuracy Study**

### **Abstract**

#### **Background:**

New cases of oropharyngeal squamous cell carcinoma (OPSCC) are routinely tested for HPV. HPV in saliva can be detected with PCR, but its clinical applicability in the context of OPSCC remains unknown.

#### **Methods**

Forty-six consecutive patients diagnosed with OPSCC had pre-treatment saliva specimens collected. PCR for HPV on saliva was compared to p16 IHC and HPV DNA in-situ hybridisation (ISH) on surgical biopsies.

#### **Results**

The sensitivity and specificity of saliva testing when compared to the reference test of p16 IHC and HPV DNA ISH was 72.2% and 90% and positive and negative predictive values were 96.3% and 47.4%. There were no adverse events. Time from last meal, smoking, alcohol drinking and physical exercise did not impact on results.

#### **Conclusions**

Saliva testing is a promising test to detect HPV in patients with OPSCC. A positive result could avoid the need for surgical biopsies, thereby reducing costs, patient morbidity, and expedite treatment.

#### 4.1 Background:

Oropharyngeal squamous cell carcinoma (OPSCC) which affects the tonsils and tongue base is traditionally associated with excess alcohol consumption and smoking. Despite a reduction in smoking and alcohol consumption, the UK incidence is rising exponentially [8]. The increase is mirrored in many developed countries [197], with approximately 63,000 new cases of OPSCC reported annually [198]. The majority are now attributed to HPV infection (HPV+ve) [197]. A worldwide meta-analysis has shown that HPV associated OPSCC has increased from 40.5% before 2000 to 72.2% after 2005 [155]. HPV+ve OPSCC is considered a major health epidemic in the western world [22].

HPV detection is emerging as a biomarker for patients with OPSCC. The presence of HPV in OPSCC can predict prognosis and determine suitability for entry into de-escalation clinical trials aimed at improving long-term quality of life [24,199]. In fact, HPV+ve and HPV-ve OPSCC are staged separately in the latest edition of the AJCC cancer-staging manual [200]. Whilst current treatments are not based on HPV status (outside of clinical trials), the new staging system is likely to have an impact.

HPV testing using p16 immunohistochemistry (IHC) on surgical or core biopsies is considered the standard of care for patients with OPSCC, and is recommended in all patients by the UK National Institute for Health and Care Excellence (NICE) [159]. p16 IHC is also the standard of care utilised by the AJCC in determining the presence of HPV in patients with OPSCC [200]. Other techniques for HPV testing including the sensitivity and specificity of these tests are described in **Table 4-1**.

**Table 4.1:** Sensitivity and specificity of clinically available tests for HPV in OPSCC (Schache et al 2011) [170].

Test	Sensitivity	Specificity
P16 IHC	94-100%	79-82%
PCR	97%	87%
DNA ISH	89%	89%

HPV testing for OPSCC is typically performed on surgically obtained biopsies performed under general anaesthesia or on core biopsies of suspected lymph node metastasis obtained under ultrasound guidance. These investigations have associated costs and risks including pain, bleeding, infection, dental injury, oesophageal perforation and rarely airway compromise. The procedures can result in delays to the patient pathway particularly when a diagnosis of squamous cell carcinoma is first made with fine needle aspiration cytology (FNAC). In these cases a supplementary surgical or core biopsy is needed from the primary site or nodal metastasis to confirm p16 status. With existing pressures on cancer diagnostic services [201], the process from initial presentation to establishing the HPV status on a biopsy of a patient with OPSCC may take weeks.

HPV is detectable in the saliva of patients with OPSCC using PCR. This non-invasive, relatively inexpensive test, without side effects and taken at the point of first patient contact could allow for the HPV status of patients to be established much earlier. Salivary HPV testing has the potential to mitigate the need for invasive biopsies performed solely for the purpose of HPV detection, and could form a routine part of screening and risk stratification in patients suspected of OPSCC. To our knowledge, there is no prospective diagnostic accuracy study directly comparing the efficacy of oral rinse testing to the clinically accepted standards of p16 IHC and DNA ISH in patients with OPSCC.

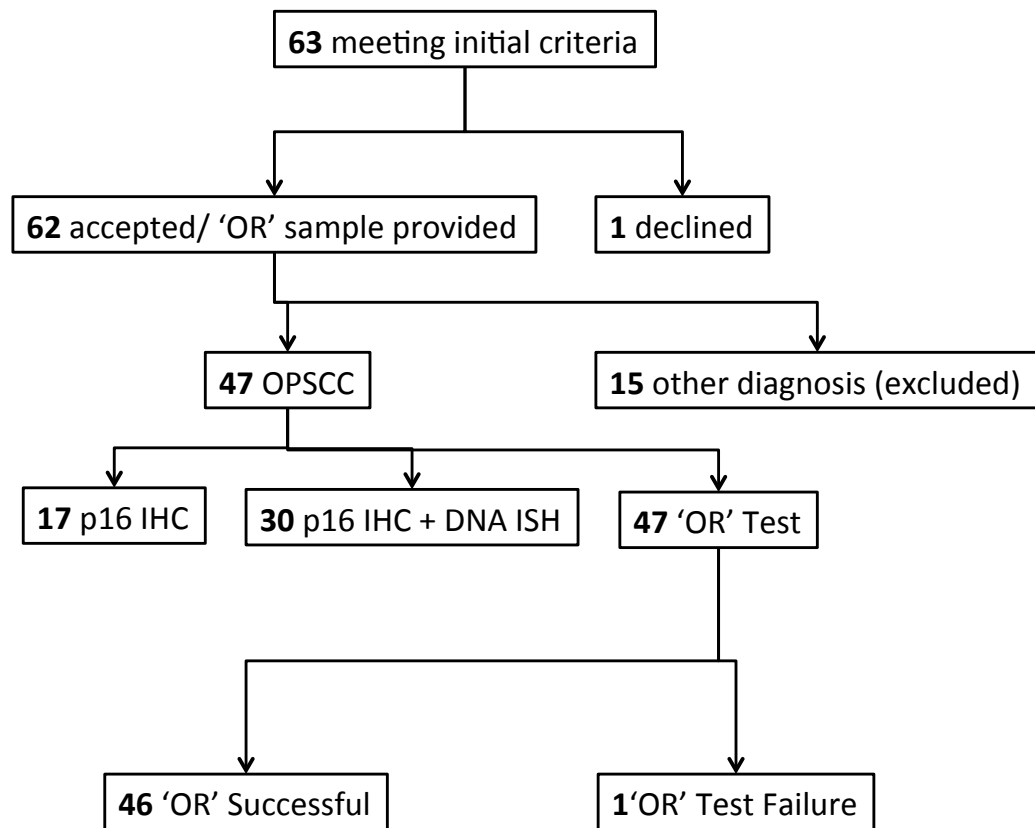
#### **4.2 Aims:**

The aim of this study was to determine the sensitivity and specificity of oral rinse (OR) testing for HPV in patients with OPSCC.

#### **4.3 Methods:**

A prospective diagnostic accuracy study estimating the sensitivity and specificity of oral rinse testing using PCR for HPV to p16 IHC and DNA ISH in patients with OPSCC was conducted. The study flow diagram is shown in **Figure 4-1**. Both QUADAS-2 [202] and STARD criteria [203] were adopted. Ethical approval was sought in collaboration with the Oxford Radcliffe Biobank (Ref No. 09/H0606/5+5).

**Figure 4-1: Study Flow Diagram – Oral rinse testing for HPV in OPSCC**



Abbreviations: OR, oral rinse

Between September 2015 and June 2016, sixty-three patients were referred to the Oxford University Hospitals (OUH) Head and Neck Cancer Service with suspected OPSCC. Consecutive adult patients (aged  $\geq 18$ ) with mental capacity and suspected OPSCC were invited to participate. All patients met initial inclusion criteria, one patient declined consent. Oral rinse (OR) specimens and demographic data were collected from the 62 consenting patients prior to commencing diagnostic assessment and cancer treatment. 47 patients were subsequently confirmed to have a diagnosis of OPSCC and were included in this study.

A standard operating procedure was developed whereby patients were asked to ‘gargle’ 10mls of sterile sodium chloride solution for 20-30 seconds. This was collected in a sterile universal container and stored within 2 hours of collection at  $-80^{\circ}\text{C}$ . OPSCC tumour biopsies were performed under general anaesthesia and formalin fixed, paraffin embedded tissue was assessed for routine diagnostic histopathology. Immunostaining for p16 was performed on all cases and confirmatory HPV DNA ISH on twenty-nine. HPV DNA ISH was performed when felt to be of clinical value, this was at the discretion of the reporting Consultant Head and Neck pathologist.

p16 IHC was considered positive when  $>70\%$  of cells showed strong nuclear and/or cytoplasmic staining [159]. HPV DNA ISH was carried out using the Ventana INFORM HPV III Family 16 Probe (B) (MDCI Ltd, West Sussex UK). The probe cocktail has demonstrated affinity to the following genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66.

The index test (*Linear Array HPV Genotyping Test Kit – Roche ©*) was performed by the OUH Molecular Diagnostics Department in accordance with the manufacturers instructions on all 47 samples. HPV DNA was extracted from each OR sample by lysing cells in denaturing conditions at elevated temperatures. PCR amplification for 37 different HPV subtypes was performed using the provided ‘master mix’. Hybridization was used to label oligonucleotide probes before using a Streptavidin-Horseradish peroxidase conjugate to identify HPV status and sub-type using the Linear Array HPV Genotyping Test Reference Guide. Suitable controls were used at each stage of the process. There was one OR test failure meaning that 46 of 47 eligible patients were included in the final analysis. Both reference and index tests were conducted by qualified technicians blinded to the clinical presentation and results.

When the results of p16 IHC and DNA ISH were combined to determine HPV status a sample was considered positive if it was either p16 IHC positive with no HPV DNA ISH test, p16 IHC positive and HPV DNA ISH positive or p16 IHC negative and DNA ISH positive. A sample was considered HPV negative if it was p16 IHC negative with no HPV DNA ISH test, p16 IHC negative and HPV DNA ISH negative or p16 IHC positive and DNA ISH negative.

### Statistical Analysis

A power calculation was conducted using Stata 13 (StataCorp LP) software to determine a sample size of 45 for 80% power to determine results with a standard error of 5% and 10% confidence intervals. All statistical analyses were pre-determined and performed using Prism 6 for Mac OS X (© 1994-2015 GraphPad Software, Inc). The demographic differences in the patient population based on p16 IHC and ‘true vs. false’

OR results were analysed using a Fisher's exact test when categorical values were less than 5 and a Chi<sup>2</sup> test when they were greater than 5 or when there were more than 2 categories. A result was considered significant when p was  $\leq 0.05$ . Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and their 95% confidence intervals were calculated.

#### **4.4 Results:**

The mean age of participants was 58.8 years (range; 37-80) (see **Table 4-2**). There were 35 males (76.1%) and 11 females (23.9%); all patients were of Caucasian descent. The mean alcohol consumption was 19.8 units per week; there were 34 (73.9%) current or ex tobacco smokers with an average 23.2 pack year (range; 1-50) smoking history. The majority of patients presented with T1/2 tumours (71.7%) with advanced local metastases in 78.3% (N2/3). One patient (2.2%) had distant metastases. Five patients had Stage 1, 28 Stage 2 and 13 Stage 3 disease (ICON-S).



**Table 4-2: Patient Demographics and Tumour Characteristics by p16 Status**

<b>Tumour HPV Status, No.(%)</b>				
	Total Patients, No.(%) N=46	p16 +ve, No.(%) (n=34)	p16 -ve, No.(%)(n=12)	P- Value
<b>Sex</b>				
Male	35 (76.1)	25 (73.5)	10 (83.3)	0.70
Female	11(23.9)	9 (26.5)	2 (16.7)	
<b>Age, (years)</b>				
>60	18 (39.1)	12 (35.3)	6 (50)	0.37
≤60	28 (60.9)	22 (64.7)	6 (50)	
<b>ASA grade</b>				
1/2	42(91.3)	32(94.1)	10(83.3)	0.28
3/4/5	4 (8.7)	2(5.9)	2(16.7)	
<b>Ethnicity</b>				
Caucasian	46 (100)	34 (100)	12 (100)	n/a
<b>Drinking Status</b>				
Nil	6 (13)	5 (14.7)	1 (8.3)	0.12
1-21 units/week	29 (63)	22 (64.7)	7 (58.3)	
21-40 units/week	7 (15.2)	6 (17.6)	1 (8.3)	
>40 units/week	4 (8.7)	1 (2.9)	3 (25)	
<b>Smoking status</b>				
Never	12 (26.1)	10 (29.4)	2 (16.7)	0.44
Ex-smoker	21 (45.7)	16 (47.1)	5 (41.7)	
Current	13 (28.3)	8 (23.5)	5 (41.7)	
<b>T Classification</b>				
T1/2	33 (71.7)	25 (73.5)	8 (66.7)	0.71
T3/4	13 (28.3)	9 (26.5)	4 (33.3)	
<b>N Classification</b>				
N0/1	10 (21.7)	5 (14.7)	5 (41.7)	0.05
N2/3	36 (78.3)	29 (85.3)	7 (58.3)	
<b>M classification</b>				
M0	45 (97.8)	33 (97.1)	12 (100)	1.00
M1	1 (2.2)	1 (2.9)	0 (0)	

Abbreviations: n/a, not applicable; ASA, American Society of Anaesthesiologists Performance Status.

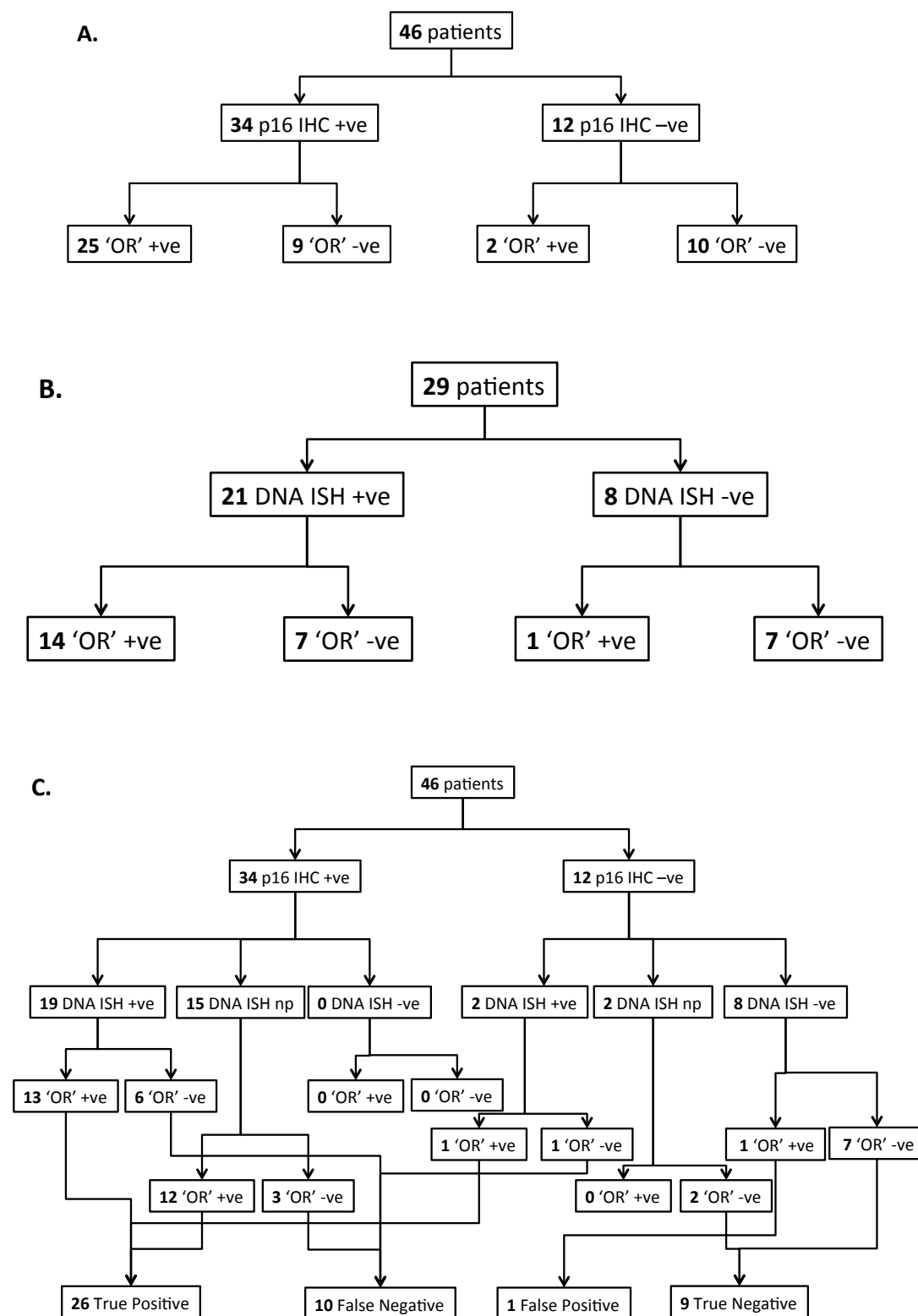
There were 34 (73.9%) p16 positive (+ve) and 12 (26.1%) p16 negative (-ve) samples. There were no statistically significant differences between the two groups in terms of age, sex, co-morbidities, alcohol consumption, smoking status, tumour size or distant metastases. Patients with p16+ve OPSCC presented with a higher N stage than those that were p16-ve (85.3% vs. 58.3%  $p=0.05$ ).

#### 4.4.1 Disease Prevalence, Sensitivities, Specificities and Predictive Values for HPV

The patient specimens were categorised into 3 groups for analysis: those that had p16 IHC and OR testing ( $n=46$ ), those with DNA ISH and OR testing ( $n=29$ ) and those that had p16 IHC with or without DNA ISH and OR testing ( $n=46$ ). The patients and test results for each group are summarised in **Figure 4-2** and **Table 4-3**.

**Figure 4-2: Results of HPV Testing**

**A**, Patients tested with p16 IHC and ‘oral rinse test (OR)’; **B**, Patients tested with HPV DNA ISH and ‘oral rinse test’; **C**, Patients tested with p16 IHC without or without DNA ISH and ‘oral rinse test’.



Abbreviations: OR, oral rinse test; +ve, positive; -ve, negative

**Table 4-3:** Sensitivity, Specificity, Disease Prevalence and Predictive Values of 'oral rinse testing' against clinical standard tests of (A) p16 IHC (B) HPV DNA ISH and (C) Combined p16 IHC/DNA ISH

	HPV Prevalence (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
<b>A</b> 'oral rinse' vs. p16 IHC, % (95% CI)	73.9(58.9-85.7)	73.5(55.6-87.1)	83.3(51.6-97.9)	92.6(75.7-99.1)	52.6(28.9-75.6)
<b>B</b> 'oral rinse' vs. HPV DNA ISH, % (95% CI)	72.4(52.8-87.3)	66.7(43-85.4)	87.5(47.4-99.7)	93.3(68.1-99.8)	50.0(23.0-77.0)
<b>C</b> 'oral rinse' vs. p16 IHC +/- HPV DNA ISH, % (95% CI)	78.3(63.6-89.1)	72.2(54.8-85.8)	90(55.5-99.8)	96.3(81.0-99.9)	47.4(24.5-71.1)

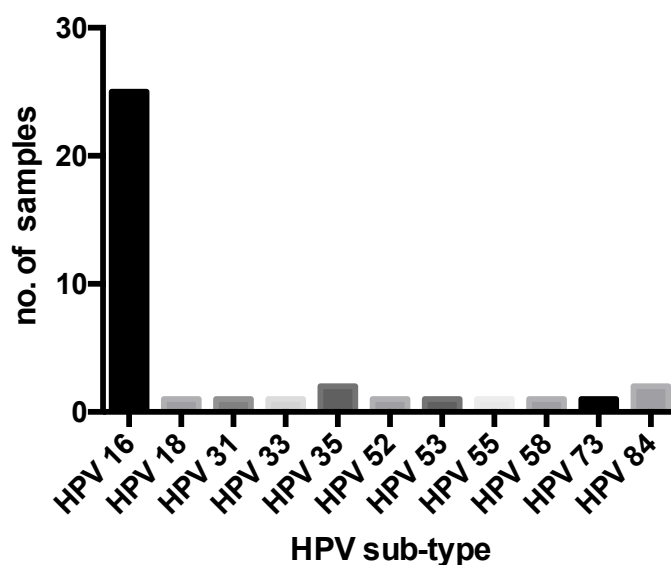
Abbreviations: CI, confidence interval; HPV, human papilloma virus; DNA ISH, DNA in situ hybridisation; p16 IHC, p16 immunohistochemistry; PPV, positive predictive value; NPV, negative predictive value.

Using the previously described method for combined p16 IHC and DNA ISH as the reference test there were 36 HPV positive (78.3%) and 10 HPV negative (21.7%) samples. Of the 36 HPV positive samples 26 were positive and 10 negative on OR testing. Out of the 10 HPV negative samples 1 was positive and 9 negative on OR testing. The prevalence of HPV was 78.3% (63.6%-89.1% 95% CI). The sensitivity and specificity of OR testing when compared to the reference test of p16 IHC with and without HPV DNA ISH was 72.2% (54.8%-85.8% 95% CI) and 90% (55.5%-99.8% 95% CI) and PPV and NPV were 96.3% (81.0%-99.9% 95% CI) and 47.4% (24.5%-71.1% 95% CI).

#### 4.4.2 Identification of HPV Sub-types

Oral rinse testing using the Linear Array HPV Genotyping Test Kit (Roche ©) allows identification of up to 37 different HPV subtypes. Twenty-seven out of 46 ‘oral rinse’ tests were positive for HPV (see **Figure 4-3**). There was 1 false positive result that suggested the presence of HPV 16 (p16 IHC and HPV DNA ISH negative). HPV 16 alone was detected in 21 samples (77.8%); HPV 18 alone in 1 sample (3.7%), whilst the remaining 5 samples (18.5%) all contained HPV 16 alongside other HPV subtypes. The combinations detected were HPV 16/33/35/52/58, HPV 16/31/35, HPV 16/53, HPV 16/84 and HPV 16/55/73/84.

**Figure 4-3:** Frequency of HPV subtypes detected by ‘oral rinse’ testing in p16+ve tumours



#### 4.4.3 Evaluating Potential Confounding Factors

To evaluate potential patient demographic, tumour or environmental factors that may have resulted in a false test result additional patient specific information was collected including time of last alcoholic drink, smoke, meal and exercise (see **Table 4-4**). Using the reference standard of combined p16 IHC and HPV DNA ISH as previously described there were 35 patients (76.1%) with a true positive or negative result and 11 (23.9%) with a false positive or negative result. There was no statistically significant difference between the two groups in terms of age, co-morbidities, T/N/M stage, last meal/smoke/alcohol or exercise.

**Table 4-4:** Comparing patient differences between ‘True’ and ‘False’ results

Oral Rinse Test Result				
	Total Patients, No.(%) N=46	True +ve/-ve, No.(%) (n=35)	False +ve/-ve, No.(%) (n=11)	P- Value
Age, (years)				
>60	18(39.1)	16(45.7)	2(18.2)	0.16
≤60	28(60.9)	19(54.3)	9(81.8)	
ASA				
1/2	42(91.3)	31(88.6)	11(100)	0.56
3/4/5	4(8.7)	4(11.4)	0(0)	
T Classification				
T1/2	33(71.7)	24(68.6)	9(81.8)	0.47
T3/4	13(28.3)	11(31.4)	2(18.2)	
N Classification				
N0/1	10(21.7)	8(22.9)	2(18.2)	0.99
N2/3	36(78.3)	27(77.1)	9(81.8)	
M classification				
M0	45(97.8)	34(97.1)	11(100)	0.99
M1	1(2.2)	1(2.9)	0(0)	
Last Alcoholic Drink				
Never	16(34.8)	12(34.3)	4(36.4)	0.82
< 6 hours	1(2.2)	1(2.9)	0(0)	
6-24 hours	20(43.5)	16(45.7)	4(36.4)	
> 24 hours	9(19.6)	6(17.1)	3(27.3)	
Last Smoke				
Never	34(73.9)	26(74.3)	8(72.7)	0.40
< 6 hours	7(15.2)	4(11.4)	3(27.3)	
6-24 hours	2(4.3)	2(5.7)	0(0)	
> 24 hours	3(6.5)	3(8.6)	0(0)	
Last Meal				
< 2 hours	7(15.2)	4(11.4)	3(27.3)	0.30
2-6 hours	25(54.3)	21(60)	4(36.4)	
> 6 hours	14(30.4)	10(28.6)	4(36.4)	
Last Exercise				
< 6 hours	4(8.7)	4(11.4)	0(0)	0.44
6-24 hours	10(21.7)	8(22.9)	2(18.2)	
> 24 hours	32(69.6)	23(65.7)	9(81.8)	

Abbreviations: n/a, not applicable; ASA, American Society of Anaesthesiologists Performance Status.

#### 4.4.4 Adequacy of Samples and Adverse Events

One (2.1%) of 47 oral rinse samples provided a non-interpretable result. The sample was analysed twice giving the same result. In accordance with the manufacturers instructions an invalid result was believed to be due to inadequate specimen collection which may have related to the collection of saliva, sample processing e.g. prolonged processing time, or the presence of inhibitors e.g. activity of enzymes. Repeat sampling of the same patient was recommended by the product manufacturer but not possible as treatment for this patient had already commenced by this stage.

Whilst some patients reported that the taste of sodium chloride was unpleasant and some needed to repeat the test due to spillage, there were no adverse events associated with oral rinse testing.

#### **4.5 Discussion:**

Our study has demonstrated that OR testing using PCR to detect HPV in patients with OPSCC has sensitivity, specificity, positive and negative predictive values of 72.2%, 90%, 96.3% and 47.4% respectively. Whilst the NPV is low making interpreting a negative result unreliable, with one sample failure and no adverse events reported this study demonstrates that PCR on oral rinse specimens is a viable method for determining HPV in OPSCC. Smoking, alcohol consumption, last meal and exercise do not appear to affect the accuracy of OR testing meaning that specific pre-test preparation is not required.

Testing for HPV in OPSCC is increasingly important as HPV status can provide important diagnostic and prognostic information. Testing needs to be sensitive and



specific, reproducible and validated. There is considerable debate as to the optimum test for HPV [170]. The commonly described techniques of p16 IHC, HPV DNA ISH and PCR on tumour tissue have associated advantages and disadvantages [204]. HPV DNA ISH is able to demonstrate HPV integration within tumour cells, but as it detects DNA and not mRNA it also does not confirm transcriptional activity [204]. p16 IHC, although not 100% specific is emerging as a prognostic marker for OPSCC in its own right [205,206]. It is considered a surrogate marker of HPV transcriptional activity[204]. There are associated limitations, Wasylyk et al (2013) [169] reported p16 IHC alone confirmed a significant number of false positives for HPV when compared to p16 IHC supplemented by either PCR or DNA/RNA ISH. PCR for HPV is highly sensitive; whilst this has obvious advantages it is not always possible to tell if the DNA detected relates to the tumour cells or surrounding normal tissue [204].

OR testing offers an alternative to conventional HPV testing in OPSCC with potential benefits of reduced patient morbidity, shorter time to diagnosis and reduced cost. It is also limited by the highly sensitive nature of PCR, whereby detected HPV DNA might not relate to tumour tissue, and instead represent a bystander infection.

The sensitivity of salivary testing however has been questioned with some authors reporting an inability to detect HPV from OR specimens in patients with known HPV+ve OPSCC [191]. Zhao et al [207] detected HPV in 50% (21 of 42 specimens) of salivary samples from patients with HPV+ve OPSCC using PCR. Whilst Wang et al [192] found that saliva was inferior to plasma when testing for HPV in patients with OPSCC. They detected HPV in 40% of their salivary specimens and 86% of their plasma specimens. Our results contradict these findings; whilst plasma testing for HPV

was not performed we were able to detect HPV in the saliva of HPV+ve cases in 72.2% of cases. This could relate to the way samples were collected, processed and stored in the studies.

Although the results of our study, which to our knowledge is the first of its kind, are promising, it is important to consider the results of PCR testing for HPV using other non-invasive biopsies. Channir et al [181] recently utilised PCR on fine needle aspirates (FNAs) of patients with OPSCC reporting 94.7% sensitivity and 100% specificity whilst Brogile et al [183] used PCR on oropharyngeal brush cytology specimens from patients with OPSCC reporting sensitivity and specificity values of 83% and 94%. Although these results seem superior to OR testing, FNA assessment and brush cytology analysis have limitations. Specifically FNAs are limited by the quality of the sample obtained which may be operator dependant [208] whilst inadequate sampling from oropharyngeal brush cytology is high (45.7%) [185]. Both tests require further clinical validation.

Unlike other diagnostic accuracy studies utilising non-invasive biopsies to determine HPV status in OPSCC this study was carried out prospectively using QUADAS-2 and STARD criteria to limit bias and mirror clinical practise. Patients were included consecutively, analyses predetermined and participants closely reflected those seen in clinical practise e.g. HPV prevalence >70%, no involuntary exclusions. Both reference and OR tests were performed by qualified technicians blinded to the study objectives and other test results to limit bias.

This study was limited by the lack of E6/7mRNA testing considered the research ‘gold standard’ for HPV detection in OPSCC [175]. Whilst the clinical standards of p16 IHC and DNA ISH were utilised as reference tests all included samples did not have HPV DNA ISH performed. This could have introduced bias in the results and underestimated the specificity and negative predictive value of OR testing. Nonetheless, the reference test utilised represented clinically acceptable standards for HPV detection in OPSCC. Separate analyses were performed for p16 IHC alone and p16 IHC supplemented by DNA ISH when clinically indicated.

#### **4.6 Conclusions:**

Given the negative impact of delayed diagnosis and treatment, OR testing could allow for immediate HPV testing at the primary clinical presentation even before specialist consultation. The relatively high specificity and PPV and low NPV mean that a positive OR test could be relied upon whilst a negative test would require additional conventional testing. This would only apply to the population of patients suspected of having OPSCC, given the high prevalence of the disease (as seen in the present study and previously reported in the literature [199]).

General practitioners could take samples from patients at the time of specialist referral so that HPV status could be available at the first specialist assessment. Patients presenting with an oropharyngeal lesion and neck nodes (as in most cases of OPSCC) could then have a core biopsy performed at the specialist consultation followed by cross-sectional imaging for staging. These results could provide the treating clinician with a tissue diagnosis, HPV status (if OR positive) and radiological stage. In selected cases, for example when a patient is deemed medically unfit for surgery, this could be

sufficient to plan treatment and avoid the risks and time associated with surgical biopsies. In select cases e.g. T1/T2 OPSCC with good mouth opening, this could also allow sufficient information for surgical planning in the form of transoral robotic resection.

Given the relatively low prevalence of high risk HPV subtypes in the general population (1%) [156], and the fact that we are unclear as to which infections are likely to persist, OR testing is unlikely to have a clinical role in HPV related OPSCC screening for the general population. The diagnostic accuracy of this test will be too low given a 1% prevalence of the disease. However, in patients with HPV associated OPSCC where the prevalence is high, OR testing might provide an opportunity for post treatment monitoring of recurrence. In fact, given that the prevalence of HPV will be 100% in this population, the diagnostic accuracy for OR testing is likely to be even higher than reported in the present study.

Furthermore, given that most cases of recurrence are within the first year after treatment [209] and the high PPV of the test in this population of patients, regular OR tests might provide earlier detection of recurrence. For example, patients might have a post treatment OR test, followed by monthly tests at their clinical follow up appointments.

In theory, this would provide information prior to any repeat imaging or clinical examination findings. In fact, post treatment positive OR tests might suggest the microscopic persistence of malignant disease requiring further treatment that might otherwise go undetected till the disease has advanced. At present there are no post

treatment diagnostic techniques in practise offering this level of monitoring that are safe and easily administered.

Overall, there is a need for large prospective studies that look not only at the diagnostic accuracy of HPV detection in OPSCC but also at recurrence and the role of HPV testing in surveillance. It may be that OR testing alone may not be sufficient and require combination with other non-invasive tests. Ahn et al [195] recently tested both saliva and serum to detect HPV in recurrent OPSCC with >90% specificity and 69.5% sensitivity. Future studies should compare all non-invasive diagnostic materials for HPV including brush cytology, FNA and serum plasma to both clinical and research standards for HPV detection e.g. DNA ISH, RNA ISH, p16IHC and HPV DNA PCR.

## **Chapter 5: Conclusions - Molecular Targets in Head and Neck Cancer**

Treatments for HNSCC have failed to keep pace with treatments for other solid organ tumours e.g. breast cancer and malignant melanoma. For breast cancer and malignant melanoma, personalised therapies are a reality and have demonstrated improved patient survival. This thesis explored some of the molecular targets described in HNSCC, aiming to answer specific clinical questions relating to the management of HNSCC.

For early LSCC, where radiotherapy is often first line treatment, radiotherapy failure and the subsequent morbidity of salvage laryngectomy remain an issue. There is a clinical need to identify a biomarker of radiotherapy failure in LSCC. This could avoid the morbidity of radiotherapy and salvage laryngectomy in appropriately selected patients and potentially improve survival by the use of alternative primary treatments e.g. TLM or radiosensitizing agents e.g. RTKs.

In OPSCC, HPV has shown promise as a prognostic biomarker and potential guide for de-escalation treatment, however controversies over the ideal method for its detection have arisen. HPV can be detected in tumour biopsies using a variety of techniques, other non-invasive sampling methods have also been described e.g. saliva. There is a clinical need to evaluate the diagnostic accuracy of these techniques before they are utilised in clinical practise.

This thesis aimed to explore the role of IGF-1R as a predictor of radiotherapy resistance in LSCC and evaluated the diagnostic accuracy of salivary testing for HPV in OPSCC.

### 5.1 IGF-1R and Radiotherapy Resistance in Laryngeal Cancer

To explore the potential role of IGF-1R as a marker of radiotherapy resistance in LSCC, tumour biopsies were collected from patients that had and had not responded to radiotherapy. IHC was used to compare levels of IGF-1R and EGFR between the two groups.

The results demonstrated higher levels of IGF-1R in LSCC biopsies from patients with radiotherapy failure when compared to those with no known recurrence following radiotherapy. They also showed rising levels of IGF-1R in patients with radiotherapy failure when comparing initial and post radiotherapy biopsies, suggesting an association between radiotherapy treatment and increased IGF-1R levels. A weak association between IGF-1R and EGFR levels was also noted.

These results provided the basis for subsequent cell line work looking at the potential for IGF-1R to act as a driver of radiotherapy resistance in LSCC. Two laryngeal cancer cell lines were repeatedly irradiated in an attempt to create a relatively radiotherapy resistant phenotype. One radiotherapy resistant cell line was generated. Western blotting demonstrated higher levels of IGF-1R in the relatively radiotherapy resistant cell line, and increased IGF-1R levels following radiotherapy in the other cell lines. These results mirrored those seen in earlier IHC studies.

In one cell line, EGFR and IGF-1R cross talk was demonstrated, EGF ligand was shown to activate IGF-1R. When BI836845 (IGF-ligand antibody) was given alongside EGF ligand in an attempt to prevent activation of IGF-1R, there was no detectable effect on

signalling. These results suggested the formation of an IGF-1R/EGFR complex or signalling via an alternative pathway e.g. G-protein coupled receptors.

Drugs targeting the IGF-1R (BI836845) and EGFR (Afatinib) signalling cascades were tested as radio sensitizing agents, their combined use appeared to be no superior to their use alone. However, combined EGFR/IGF-1R inhibition appeared to have a significant impact on baseline cell survival when compared to each agent alone. The effect was greatest in previously irradiated cells with elevated IGF-1R.

Whilst the results of these studies should be interpreted cautiously, given the limitations as previously described in each chapter, the results warrant further investigation. The findings suggest an association between IGF-1R levels, radiotherapy resistance and previous radiotherapy. Providing evidence for IGF-1R as a marker of radiotherapy resistance in LSCC.

They also suggest a role for combined IGF-1R and EGFR inhibition as a potential treatment for patients with LSCC and IGF-1R overexpression. So far, drugs targeting RTKs e.g. Cetuximab have been limited by the development of treatment resistance [1], this might be due to receptor cross-talk [51], whereby one RTK compensates for the loss of another. Cross talk between EGFR and IGF-1R was clearly demonstrated in the study described and whilst combined target inhibition did not appear to prevent signalling, there was a significant effect on survival in cells with IGF-1R overexpression. These results suggest the potential for combined receptor targeting or targeted patient selection providing the solution to issues around treatment resistance.



So far only human papilloma virus (HPV) has shown promise as a biomarker for treatment in OPSCC [93]. There are no clinically utilised biomarkers for personalised therapy in LSCC. If these results are replicated in large-scale prospective studies, IGF-1R status could help refine current treatment decisions. Much like HPV testing for OPSCC, IGF-1R testing could contribute to treatment decisions in LSCC. Patients with high IGF-1R could be offered primary surgery with radiotherapy and chemotherapy held in reserve. This could alter current trends towards primary radiotherapy based on TNM status and patient factors [30] to treatment guided by the molecular profile or IGF-1R status of tumours.

Prospective large-scale multi-centre trials are needed to test these findings and determine the ‘clinically relevant’ level to define IGF-1R overexpression. There is also a need for further investigation into the interaction between EGFR, IGF-1R and other RTKs that might play a role in radiotherapy resistant HNSCC. Future studies should focus on the use of patient tissue pre and post radiotherapy and utilise drugs targeting alternative parts of the IGF-1R and EGFR signalling cascade in an attempt to radiosensitive laryngeal cancer cells.

### 5.2 HPV testing in Oropharyngeal Cancer

HPV testing for OPSCC is complex with various techniques described using FFPE tissue and ‘liquid biopsies’ e.g. saliva, brush cytology, FNA and serum. Despite the variety of available tests there is no consensus on which technique is best, although p16 IHC on FFPE tissue is considered the clinical standard [159]. To evaluate the evidence behind each of the techniques available for HPV testing in OPSCC a literature review was performed.

P16 IHC, HPV DNA ISH and HPV PCR are the commonest tests to determine HPV status. P16 IHC and HPV DNA PCR are highly sensitive whilst HPV DNA ISH is more specific; these techniques typically utilize surgical biopsies. Current evidence suggests that conventional p16 IHC is useful as a screening tool for determining HPV status, and should be considered the first line test in all cases of OPSCC. Given the findings of a recent meta-analysis, there is also evidence to support additional testing using a technique with greater specificity e.g. HPV DNA ISH or PCR [169]. There is also a need for a single modality test to increase widespread adoption and limit costs. RNA ISH has shown promise in this regard.

New tests using PCR to screen fine needle aspirates, saliva, brush cytology and serum for HPV are promising but are yet to be validated in a clinical setting. These non-invasive samples avoid the morbidity of surgical biopsies and need for tissue blocks; their clinical role in screening and surveillance remains largely untested.

To answer this important clinical question, a study was designed to determine the diagnostic accuracy of PCR on saliva to determine HPV status in patients with OPSCC. The index test was compared to the reference standards of p16 IHC with HPV DNA ISH. The overall results demonstrated 72.2% sensitivity and 90% specificity, with 96.3% and 47.4% positive and negative predictive values. The results were not influenced by pre-test meal, alcohol consumption or smoking, meaning there was no need for prior preparation. Given the negative impact of delayed diagnosis and treatment, saliva testing could allow for immediate HPV testing at the primary clinical presentation even before specialist consultation. The relatively high specificity and PPV and low NPV mean that a positive saliva test could be relied upon whilst a negative test

would require additional conventional testing.

Clinically, this means that general practitioners could take samples from patients at the time of specialist referral, so that HPV status could be available at the first specialist assessment. This is particularly important given that the latest AJCC staging criteria for OPSCC is now dependant on HPV status [200]. Patients presenting with an oropharyngeal lesion and neck nodes (as in most cases of OPSCC) could have a core biopsy performed at the specialist consultation followed by cross-sectional imaging for staging. These results would provide the treating clinician with a tissue diagnosis, HPV status (if positive) and radiological stage. In selected cases, for example when a patient is deemed medically unfit for surgery or the patient has declined surgical treatment, this would be sufficient to plan treatment and avoid the risks and time associated with surgical biopsies. Of course, there are likely to be exceptions e.g. when there is insufficient tissue or when a synchronous primary is suspected.

Despite the limitations of this study, the results provide evidence to support the design of larger more comprehensive trials investigating ‘liquid biopsies’ in detecting HPV in OPSCC.

Overall, after completing this thesis, I can make the following conclusions:

1. Elevated IGF-1R appears to associate with previous radiotherapy and radiotherapy resistance in LSCC. Treatments accounting for IGF-1R status, or molecular therapies targeting this receptor, may have merit in patients whose tumours overexpress IGF-1R.
2. Saliva testing for HPV is a promising alternative to p16 IHC performed on tumour tissue. In selected patients, this might avoid the need for surgical biopsies and expedite treatment.

## Abbreviations:

Abbreviation	Meaning
-ve	Negative
%	Percentage
+ve	Positive
1°	Primary
Af	Afatinib
AJCC	American Joint Committee on Cancer
ASA	American Society of Anaesthesiologists Performance Status
BI	BI836845
BSA	Bovine Serum Albumin
CI	Confidence Interval
CSA	Clonogenic Survival Assay
DER	Dose Enhancement Ratio
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EBV	Ebstein Barr Virus
ECACC	European Collection of Cell Cultures
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FFPE	Formalin Fixed Paraffin Embedded
FNA	Fine Needle Aspiration
FNAC	Fine Needle Aspiration Cytology
Fr	Fraction
GPCR	G Protein Coupled Receptor
GS	Goat Serum
Gy	Gray
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
IGF	Insulin Growth Factor
IGF-1	Insulin Growth Factor Ligand
IGF-1R	Insulin Growth Factor Receptor Type 1
IHC	Immunohistochemistry
IRS	Immunoreactive Score
ISH	In-situ Hybridisation
kDa	Kilodaltons
LSCC	Laryngeal Squamous Cell Carcinoma
MDT	Multi-disciplinary Team
MW	Molecular Weight
MW-U	Mann-Whitney U test
n/a	Not Applicable
NHEJ	Non Homologous End Joining
NICE	National Institute for Health and Care Excellence
nM	nanomolar
NPV	Negative Predictive Value
OPSCC	Oropharyngeal Squamous Cell Carcinoma
OR	Oral Rinse

p	Phosphorylated
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PPV	Positive Predictive Value
pRb	Retinoblastoma Tumour Suppressor Gene
r	Pearson's Correlation Co-efficient
RTKs	Receptor Tyrosine Kinases
RTOG	Radiation Therapy Oncology Group
Rtx	Radiotherapy
RwR	Radiotherapy without known recurrence
SDS-PAGE	SDS, Polyacrylamide Gel Electrophoresis
SF	Sample Failure
SF <sub>50</sub>	Dose of radiotherapy to suppress cell survival to 50% of control cells
SM	Skimmed Milk
STL	Salvage Total Laryngectomy
STR	Short Tandem Repeat
TL	Total Laryngectomy
TLM	Trans-oral Laser Microsurgery
Tx	Treatment
UK	United Kingdom
US	United States of America
VA	Veteran's Affairs

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